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California Commercial/Sport Fish and Shellfish Oil Toxicity Study. Volume 2 Synthesis of Findings

MBC Applied Environmental Sciences, Inc.
Costa Mesa, CA

Prepared for

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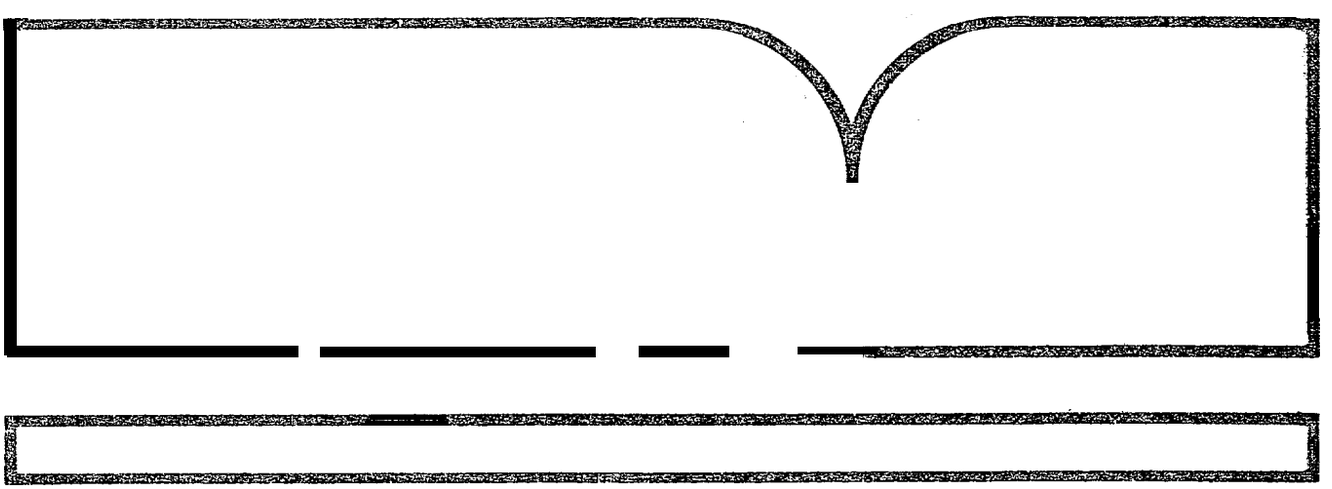

PB84- 167220

California Commercial/Sport Fish and Shellfish Oil Toxicity Study

Volume II

Synthesis of Findings

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1983

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The effect of oil on three species of California marine organisms, California Halibut (Paralichthys californicus), Northern Anchovy (Engraulis mordax), and California Mussel (Mytilus californianus) were investigated. Adult, larval, and egg life stages of each species were exposed to the water soluble fraction (WSF) of Santa F3arbara crude oil in flow-through experiments lasting through the life stage or as long as 120 days. Three concentrations of WSF were used, 5 (low), 50 (medium), and 500 (high) parts per billion (ppb) with controls in normal sea water. Behavior, growth, mortality, tissue and organ abnormalities, and tissue hydrocarbon burdens were recorded. Test solutions were monitoreddaily by gas chromatography (GC) and weekly by GC-Mass spectrometry.

Embryos of all species accumulated hydrocarbons and showed reduced survival with increasing test concentration. Larval halibut demonstrated bent notochords in high test concentration reduced growth rate in all concentrations, and gill damage in adults in high concentration Anchovy larvae and adults showed patterns similar to halibut with reduction in growth and head and jaw abnormalities. Growth of adult anchovies was reduced in high concentration. Mussel results included abnormal shell thickness, reduced byssus thread production, and significant hydrocarbon accumulation in several tissues.

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CALIFORNIA COMMERCIAL/SPORT FISH AND SHELLFISH OIL TOXICITY STUDY

VOLUME II
SYNTHESIS OF FINDINGS

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A. INTRODUCTION

1. MMS MANDATE

The Minerals Management Service (MMS) through the Pacific Outer Continental Shelf (OCS) region, is mandated by PL 95-372 (OCS Lands Act Amendment of 1978) " . ..to preserve, protect, and develop oil and natural gas resources in the Outer Continental Shelf in a manner which is consistent with the need to 1) make such resources available to meet the Nation's energy needs as rapidly as possible, 2) balance orderly energy resource development with protection of the human, marine, and coastal environments, 3) insure the public a fair and equitable return on the resources of the Outer Continental Shelf, and 4) preserve and maintain free enterprise competition."

In support of this mandate the MMS' OCS environmental studies goals are " . ..to establish information needed for prediction, assessment, and management of impacts on the human, marine, and coastal environments of the Outer Continental Shelf and the nearshore area which may be affected by oil and gas activities in such area or region" (Federal Register 43:3893, January, 1978). In pursuit of the above goals, the present oil effects study was undertaken to provide more detailed and easily applicable data on the environmental impacts of chronic oil exposure effects.

Several areas along the coast of California receive low-level chronic exposure to petroleum hydrocarbons through natural oil seepages. These natural sources are augmented by low level leakage from oil tankers, production platforms, and vessel bilge pumping. Crude oil and oil products introduced into the marine environment may come into contact with the biota either at the surface as a slick, in the water column, or in the sediment. The marine environment is dynamic, resulting in the movement of hydrocarbons between these regions, as well as out of the system.

Oil in the water column may exist as truly soluble components, or as an oil-in water dispersion. Rice et al. (1977) concluded that toxic effects are due to the chemical toxicity of soluble aromatic hydrocarbons. Eggs and larvae of vertebrates and invertebrates (e.g. crustacean larvae), are considered most sensitive to hydrocarbons in the water column (Anderson 1979). Acute toxic effects resulting in the mortality of an organism usually are manifested over a relatively short time period. Chronic effects are usually more subtle, but may affect the behavior, physiologic function, reproductive potential, or life expectancy of an organism in a way which has serious consequences at the population or ecosystem level. Because chronic effects do not immediately result in mortality, they can only be evaluated by careful laboratory bioassay.

2. STUDY OBJECTIVES

The objectives of the California Commercial and Sport Fish Oil Toxicity Study were to:

a. Determine the extent of information currently in the literature which relates to laboratory long-term oil toxicity testing on commercial/sport fish and shellfish,

b. Determine the lethal and sublethal effects of long-term exposure to low levels of crude oil on the embryo, larval, and adult life history stages of selected commercial/sport fish and shellfish.

c. Interpret the results obtained from laboratory testing as they might relate to predictions about potential or actual impacts of OCS development-of oil and gas resources on California fisheries.

3. TEST ORGANISMS

a. Test Species

The test species selected for long-term chronic exposure studies were the California halibut (Paralichthys californicus), northern anchovy (Engraulis mordax), and California mussel (Mytilus californianus). The embryo, larval, and adult life history stages of each species were studied in separate experiments. These species were considered representative of the pelagic, demersal, and nearshore habitats, respectively, in the California OCS lease area. Selection of these test organisms was based on the following criteria: 1) commercial sport and/or ecological importance in the California OCS lease area; 2) likelihood of impact by offshore oil spills; and 3) suitability as a laboratory bioassay test organism during all three life history stages.

b. Previous Work

Athough a considerable body of information presently exists concerning both the lethal and sublethal effects of hydrocarbon exposure on marine organisms, relatively few species of commercial or sport importance in California have been studied. The following sections review existing knowledge regarding hydrocarbon exposure effects on northern anchovy and California mussel. No information is presently available on California halibut.

1) California Halibut (Paralichthys californicus). No previous studies have examined the effects of oil exposure on California halibut, or closely allied species.

2) Northern Anchovy (Engraulis mordax). Struhsaker et al. (1974) exposed northern anchovy embryos to benzene at concentrations ranging between 4.0 to 55.0 ppm for periods of 24 and 48 hrs. Survival of hatched larvae at 3 and 6 days after initial exposure, respectively, was lowest at a benzene concentration of 4.7 ppm. Struhsaker et al. (1974) demonstrated that embryonic and post-hatch larval development was accelerated at 4.7 ppm benzene and retarded at 10.5 and 24.0 ppm benzene. At 53.5 ppm, surviving larvae were inactive and development was greatly delayed. At benzene concentrations <10 ppm the rate of yolk absorption was accelerated when compared with control larvae. At benzene concentrations of 24, and 40 to 55 ppm, however, yolk absorption was delayed. This pattern of yolk absorption was also reflected in the size of newly hatched larvae from benzene-exposed eggs. For example, larvae hatched from eggs exposed to 10.5 ppm benzene were larger than larvae produced by eggs exposed to either higher or lower benzene concentrations. This presumably occurred because embryos were less active, and therefore energy derived from yolk absorption was channeled into growth rather than activity.

Kern et al. (1976b) investigated the uptake, distribution, and depuration of ^{14}C-benzene by northern anchovy. Highest benzene concentrations

were accumulated in the gal I bladder, mesenteric fat, colon, intestine, and liver. The highest rate of intake occurred in the first 6 hrs, and maximum tissue concentrations were obtained within 4 days. Benzene residues were rapidly depurated after cessation of exposure, with the gall bladder, mesenteric fat, liver, and gill tissues retaining residues for the longest period.

3) California Mussel (Mytilus californianus). Toxicity studies with mollusks have been confined primarily to clams, mussels, and oysters, many of which are of sport or commercial importance in the United States, but not necessarily in California. Mytilus californianus has received limited study, and in the efforts examined considerable variation exists in the hydrocarbon tested, concentration administered, duration of exposure, and system of exposure (i.e. static versus flow-through) (Kanter 1971, 1973).

Few studies have addressed the effects of petroleum hydrocarbons (PHC) on mussel gametes and none have examined M. californianus, specifically. In experiments which concentrated on the effects of PHC on gametes, observations generally were restricted to fertilization and developmental success after various permutations of exposure. Sublethal studies are practically nonexistent. Renzoni (1973) examined the effect of water soluble fractions (WSF) from fuel and crude oil on gametes and larvae of mussels and oysters (Mytilus galloprovincial is, Crassostrea angulata, C. gigas). Normal fertilization was observed for all species in all experimental regimes, however, complete development was inhibited in high WSF concentrations (100 and 1,000 ppm) of Venezuelan and Russian crude. Sublethal developmental abnormalities noted by Renzoni (1975) in embryos exposed to WSF of Nigerian and Alaskan crude oil included enlarged embryos, variable embryo shape, and straight hinge stages which lacked the veliger shell.

Larval life stages of marine organisms represent a vulnerable critical period in the developmental life history. Though larva? studies of M. californianus are nonexistent, congeners have been examined and extrapolations can be made from this data.

Renzoni (1973) exposed fertilized oyster and mussel eggs (Crassostrea angulata, C. gigas, Mytilus galloprovincial is) to WSF of Venezuelan crude and No. 1 fuel oil. Successful larval development was assayed by noting the number of swimming larvae. Reduction in developmental success was recorded in the highest (1,000 ppm) concentration (Table II-1). Larvae developing in lower (more realistic with regards to environmental levels) concentrations of oil in water displayed success equal to that of controls.

Table 11-1. Average number (%) of larvae developed in water with oil B and with derivative E*.

	1 ppm		10 ppm		100 ppm		1000 ppm	
Control	B	E	B	E	B	E	B	E
			Crassostrea angulata					
84.6	84.3	83.6	83.3	83.6	83.0	81.3	69.3	74.0
			Crassostrea gigas					
84.3	83.6	82.3	81.6	83.3	79.3	83.6	68.6	73.0
			Mytilus galloprovinciaiis					
87.3	84.0	83.3	82.3	85.6	81.0	84.3	71.6	75.6

*from Renzoni 1973

In experiments where fertilization was allowed to take place in an oiled water environment, Renzoni (19?3) observed "first movement activity." Movement of larvae maintained in lower concentrations (1 and 10 ppm) was similar to controls, but those exposed to higher concentrations (100 and 1.000 ppm) of crude and fuel oil displayed reduced swimming speed, irregular swimming, and total inactivity.

A limited number of oil exposure experiments have been conducted on adult M. californianus. However, adult mussels of other species have been used

in several hydrocarbon studies because they are: 1) a hardy experimental test organism (Gil fillan 1973); 2) widely distributed and therefore available (Soot-Ryen 1955); 3) have been studied extensively and considerable physiological baseline data-are available (Field 1922); 4) of a convenient size, small enough to be handled easily in the laboratory and large enough for specific organ dissection (Lee et al. 1972); and 5) an important member of the intertidal ecosystem (Ricketts and Calvin 1968). As with gamete and larval studies, it is informative to consider results of experiments performed on congeneric species to supplement those reported for M. californianus.

The relative toxicities of No. 2 and No. 5 fuel oils in short-term exposure experiments on the mussel, Mytilus edulis, were investigated by Clark and Finley (1975). After exposure to surface slicks of the oils for 48 and 32 hours, mortalities of 45% and 35%, respectively, for No. 2 and No. 5 oils were recorded. Surviving animals maintained in clean seawater for up to 35 days after the experiments still contained traces of fuel oil residues in their tissues. However, no sublethal health evaluations of the surviving animals were performed.

Lee, Sauerheber, and Benson (1?72) exposed M. edulis to several individual hydrocarbon compounds including mineral oil, [^{14}C] heptadecane, 1,2,3,4 tetrahydronaphthal ene, [^{14}C] toluene, [^{14}C] naphthalene, [^{3}H] 3,4-benzopyrene for periods of 4, 6, and 76 hours. Though exposure concentrations varied, the non-toxic paraffinic hydrocarbons were generally taken up to a much greater extent (10 mg/mussel) than the aromatic hydrocarbons (2 to 20 mg/mussel). After exposure, animals placed in clean seawater discharged most of the hydrocarbons. However, the sublethal effects of the retained compounds (or latent effects of exposure) on surviving individuals was not investigated further.

Experiments by Kanter et al. (1971) examined the tolerance of - ıssels (Mytilus californianus) from "polluted" and "non-polluted" areas to high dosages (1×10^3 and 1×10^4 ppm) of Santa Barbara crude oil. Mortality was higher and occurred sooner in animals that had not been previously exposed to oil. These studies were performed in static systems for 10-day periods, but the results compare favorably with the "tolerance" recorded by Nunes and Benville (1978) in later research on the Manilla clam.

Additional mortality studies on M. californianus in static systems performed by Kanter (1974) with Santa Barbara crude spanned greater time periods. Each set of experiments lasted approximately 60 days and experimental concentrations were 1×10^3, 1×10^4, and 1×10^5 ppm total crude in water. Even with these high oil dosages, mortality in experimental animals differed between the populations tested (i.e. those animals collected from "clean" environments were more susceptible) and was dependent on size of the individuals and season. These experiments more closely approach the experimental regime desired for predictions on the California coast. However, unrealistically high oil concentrations (e.g. ppm range) and the use of a static system limit the utility of these results.

In animals exposed in the environment to natural oil seepage, deLappe et al. (1979) reported bioaccumulation of PHC in above background levels in M. californianus. These results suggest that significant deputation does not take place even when exposure to the pollutant is intermittent.

Byssus thread production by mytilids is a common, daily requirement in nature. Enhancement and/or depression of byssus thread production was

recorded by Carr and Reish (1978) in mussels (M. edul is) exposed for periods between 1 and 14 days to extracts of crude oil, No. 2 fuel oil, and outboard motor oil. Although the exposure system apparently was static, concentrations of toxicant administered were not accurately assessed.

Carbon flux as measured by respiration was depressed in mussels (M. edulis) exposed to WSF of crude oil (Gilfillan 1975). Physiological stress was also noted by Fossato and Canzonier (1976) in M. edulis exposed to diesel fuel for 15 to 20 days.

The ability of Mytilus to degrade petroleum aromatic hydrocarbons after acute exposure was investigated by Vandermeulen and Penrose (1978). After exposing Mytilus edulis to WSF of Kuwait crude or Bunker C (fuel oil) none of the animals showed evidence of hydrocarbon-induced aryl hydrocarbon hydroxylase or N-demethylase activity. The inability to produce petroleum degrading enzymes, and the tendency for PHC to bioaccumulate in tissues suggest that PHC compounds could be transferred, unaltered, into the food chain.

4. EXPERIMENTAL REGIME

a. Flow-through

Most acute toxicity testing, and much chronic toxicity testing has been performed under static conditions. A better approximation of true conditions during petroleum hydrocarbon exposure testing is the use of a flow-through system in which "new" water is constantly introduced to the system, This more nearly simulates the real-world situation where water is constantly being exchanged, and prevents static maintenance problems associated with metabolic waste build up or reduced oxygen availability. For these reasons, a flow-through system was developed and used in the larval and adult studies with each test species. Due to the small size and vulnerability to physical damage of the embryo, and short duration of this life stage, embryo (i.e. egg) experiments were conducted under static conditions.

b. Duration

All experiments were intended to be of sufficient duration so that sublethal effects of exposure could be evaluated. However, the specific time period for each experiment was different and determined by the species and life history stage tested (see Section B, Methods). The adult experiments were targeted to span a 120-day period. Larval and embryo experiments were necessarily of shorter duration than adult experiments, but were designed to be of sufficient length to assess sublethal effects. The end points of these experiments were primarily based on practical considerations related to maintenance of adequate numbers for examination.

c. Oil and Test Solution

1) Selection of Oil. Crude oils with the greatest probability of contributing to petroleum contamination in California coastal waters are those produced in the coastal and Outer Continental Shelf (OCS) areas of California. Petroleum exploration, production, and transportation activities conducted in the California OCS area may result in varying degrees of chronic petroleum contamination. Additionally, the potential for contamination through inadvertent spills is greatest for crudes produced in the California OCS area. For

these reasons, the selection of crude oil for use in this study was limited to those produced in the California coastal and OCS areas.

The area of greatest petroleum exploration, production, and transportation activity in the Pacific OCS is the Southern California Bight (Point Conception south to the U.S.-Mexican border). The most intensely developed areas within this region are centered off the coasts of Los Angeles/Orange and Santa Barbara/Ventura counties. Crude oils produced in these two offshore areas can be classified as either asphalt-based or mixed-base crudes. Although clear distinctions between these generic classifications are often lacking for a particular crude, those produced in the area offshore from Los Angeles are generally characteristic of asphalt-based oils, while those produced offshore from Santa Barbara area more closely resemble mixed-base crudes. Of these two basic types of Pacific OCS crude, Santa Barbara crude oil was selected for use in the chronic bioassay studies.

Santa Barbara crude is a mixed-base crude characterized by high sulfur content, diverse aromatic character, and a typical pattern of straight and branched chain aliphatic components. As such, Santa Barbara crude has a chemical composition very similar to that of Middle Eastern, Alaskan, and Gulf coast crudes (see Discussion). Because each of these crudes is produced and/or transported in U.S. coastal waters, and may potentially be involved in contamination of the marine environment, results of these bioassay studies should have universal applicability. Careful extrapolation of these findings to contamination/spill events may provide a means of evaluating the biological impact of chronic exposure on marine organisms.

2) Water Soluble Fraction (WSF) as a Toxicant. One approach to studying the WSF has been the use of a single petroleum hydrocarbon that is known to be a large contributor to the WSF (e.g. naphthalene, a common component of most crude oils). Use of such single sources allows the toxicant to be defined and measured very accurately, and any effects resulting from exposure of animals can then be specifically associated with the tested chemical. Such applications make utilization of single chemical testing attractive, but, as with any artificial system, some loss of information can also occur. In a real-world situation it is crude oil or some other complex petroleum hydrocarbon mixture rather than single chemicals that is most often spilled. The WSF which animals are usually exposed to contains a composite of aromatics leached from the spilled crude. Even if a given chemical is a large component of the WSF, the toxicity or sublethal effects of that component may not exactly mirror the effects of the complex WSF. Because of the potential for synergistic and/or antagonistic interactions, the total WSF was chosen for use in all chronic exposure studies.

d. Oil Dosing

1) Ambient Hydrocarbon Levels in the Marine Environment. Several investigations have been undertaken in recent years to establish the "background" level of hydrocarbons in marine waters. However, the inherent natural variability and wide range of methods used for sample collection and analysis have made correlation and evaluation of these data difficult.

The most thorough studies of background hydrocarbon levels in marine waters have been conducted in the Atlantic and Pacific Oceans and the Gulf of Mexico. These studies have largely focused on non-volatile hydrocarbons with chain lengths of greater than 12 to 14 carbon atoms.

The hydrocarbon species most frequently identified include saturated and unsaturated aliphatics (both straight and branched chain), oxygenated heterocyclics and aromatics. Results of these studies show variations in total non-volatile hydrocarbon levels ranging from approximately 0.2 to 20 µg/l. A summary of data from some of the more recent studies is presented in Table II-2. In addition to hydrocarbon levels, this table summarizes information concerning sampling locations and techniques used for sample collection and analysis.

Table II-2. Summary of investigations on ambient marine hydrocarbon levels.

Sample Collection, Extraction and Analysis Techniques	Collection Location	Hydrocarbon Species Studied and Concentration Data	Reference
401 water volumes; 0.45µ filtration; analysis by combination of liquid chromatography (LC), thin- layer chromatography (TLC), gas chromatography (GC), and infrared spectrophotometry (IR)	Gulf of Mexico and Pacific B-2780 m depth	dissolved paraffinic hydrocarbons; 820-950 µg/l; identification of C10. C12, C14. C16, and C18 paraffins	Jeffrey et al. 1964
Niskin bottle sampling; 0.45. filtration; chloroform extraction; TLC, IR, GC and mass spectrometry (MS)	Mediterranean Sea coastal waters	dissolved hydrocarbons; 47µg/l mean cone.; identification of C16, C20, and C26 n-paraffins	Coplin and Barbier 1971
Collection of subsurface waters (20 cm) using a teflon bottle; chloroform extraction; TLC and GC	Narragansett Bay, Rhode Island	mean cone. . 6 µg/l	Duce et al. 1972
Continuous extraction of unfiltered samples; Gc	Gulf of Mexico	non-volatile n-paraffins; surface water· 0.087-0.2 µg/l and deep water (2000 m): 0.78µg/l surface water: 1.1 µg/l	Parker et al. 1972
Metal bottles; 0.45µ filtration; chloroform extracting; TLC, IR, CC, MS	Manche and Mediterranean coastal equatorial Atlantic open sea	46-137 µg/l and 75µg/l, respectively. 10-43 µg/l	Barbier et al. 1973
Collection of surface samples; no filtration, carbon tetrachloride extraction; IR, MS	U.S. Gulf coast	total non-volatile hydrocarbons; 1-12 µg/l	Brown et al. 1973
Niskin bottles; unfiltered; carbon tetrachloride extraction; fluorescence	Gulf of St. Lawrence	dissolved and particulate hydrocarbons; 1-10 µg/l throughout water column for dissolved hydrocarbons	Levy and Walton 1973
Niskin bottles; Whatman No. 1 filtration; chloroform extraction; GC	Gulf of Mexico Cariaco French Caribbean Sea	dissolved non-polar hydrocarbons; traces to 75 µg/l 5 µg/l B µg/l	Iliffe and Calder 1974
Glass flask; unfiltered; chloroform methylene chloride extraction; TLC, GC	Sargasso Sea	total non-volatile hydrocarbons; 13-239 g/l: mean 73µg/l	Made and Quinn 1975
Niskin bottles; Whatman GF/C filtration; chloroform extraction; TLC, Gc	English Channel Mediterranean West Africa	dissolved and particulate n-alkanes; diss. -0.1 µg/l, part. -0.3 µg/l; diss. -0.7-2.4 µg/l, part. -0.4 µg/l diss. -5.7 µg/l, part. 0.3 µg/l	Marty and Saliot 1976
Various samplers; unfiltered; solvent extraction; IR, GC	Atlantic Pacific	3.3-13 "g/l 0.8-2.0 µg/l	Brown and Huff man 1976
Niskin bottles; Whatman GF/C and 0.45. filtration; pentane and benzene extraction; GC	Sargasso Sea	particulate hydrocarbons and tar balls; 0.7-4.4 µg/l	Morris et al. 1976
Glass bottle; unfiltered; pentane extraction; fluorescence, LC, GC	Bedford Basin Scotian shelf Gulf of St. Lawrence Sargasso Sea	total non-volatile n-alkanes; 0.07-0.6 µg/l 0.08-0.15 µg/l 0.02-1.01 µg/l 0.05-0.15 µg/l	Keizer et al. 1977
Steel container; 400 µm filtration; chloroform extraction; TLC &GC	Jeddore Harbour, Nova Scotia	total n-alkanes up to C21; 1-10 µg/l	Paradis & Ackman 1977
Glass bottles; Gilman A filtration, basic methanol extraction; GC	Narragansett Bay	total particulate hydrocarbons at the sea surface; 3-219µg/l	Schultz & Quinn 1977
In situ sampling apparatus: 0.45µ filtration; methylene chloride extraction; GC	Pacific-Southern California Bight	total non-volatile hydrocarbons from 10 locations; dissolved: 0.01- 1.1 µg/l	de Lappe et al. 1980

2) Hydrocarbon Levels in the Vicinity of Petroleum Spills. Investigations of marine oil spill incidents have focused almost exclusively on examination of non-volatile hydrocarbons. Relatively little work has been conducted on the volatile, lower molecular weight species. Such compounds are of particular importance, however, since they--include highly volatile aromatics which are relatively water soluble and highly toxic components of crude oil. Because many past studies have not examined these relatively soluble and volatile components, the measured and reported hydrocarbon levels must be considered conservative estimates of the total hydrocarbon levels present.

Water column hydrocarbon levels in the vicinity of petroleum spills have generally been observed to range from 5 to 400 µg/l, although a great deal of variation exists. Significant differences in water column hydrocarbon levels have been observed to occur at the same sampling location, as well as among different locations within a given spill area. Variations in reported water column hydrocarbon concentrations are a function of: 1) the fractions analyzed; 2) the type of material spilled; 3) the mechanism by which it is released; 4) the amount released; and 5) dynamics of the marine system in the area of release.

3) Test Solution Concentrations. Published data on water column hydrocarbon levels observed under oil spill conditions were the primary criteria used in the selection of exposure concentrations in the chronic bioassay studies. A primary objective was to design a realistic exposure regime, thus optimizing the utility of the resultant data for predictive purposes. With these design criteria in mind, target hydrocarbon exposure levels of' 5 (low level), 50 (medium level), and 500 µg/l (high level) were selected.

In many instances 500 µg/l may be 'representative of hydrocarbon levels in the immediate vicinity of an oil spill , whereas 50 µg/l and 5 µg/l more closely approximate hydrocarbon levels at greater distances from the central spill or chronic background levels. The experimental design thus provides a means of estimating potential biological impact at several zones of influence in the vicinity of the main spill.

Additional factors considered during selection included: 1) the levels of hydrocarbons projected for the OCS region off southern California in the upcoming years; 2) the variability of WSF Santa Barbara crude and problems inherent in this variability that affect maintenance of consistent experimental concentrations; 3) the sensitivity of instrumentation utilized in monitoring and analysis; and 4) the practicality of continuous production of test solution from crude oil.

B. METHODS

1. TOXICANT ADMINISTRATION AND MONITORING

a. Oil Solubilizer

Continuous dosing of single compounds (e.g. benzene) can be accomplished rather easily by the administration of stock solutions through a commercially available dosing apparatus. Working with single compounds presents fewer problems than those associated with complex hydrocarbon mixtures such as crude oil. In particular, the differential solubilities of crude oil components,

and the physical characteristics of the parental crude interact to create variables which must be controlled.

Selection of Santa Barbara crude oil WSF as the test solution necessitated design and construction of a system to produce the toxicant. Conceptual design of a solubilizer for the MMS experiments required that: 1) the solubilizer be capable of handling a viscous crude oil; 2) the solubilizer be capable of providing a continuous supply of the WSF; 3) contamination of the WSF by emulsified oil droplets be controlled; 4) the WSF concentration produced by the solubilizer be sufficiently high to meet required experimental dosage levels; 5) the system be capable of extended operation with limited maintenance.

Initially, a solubilizer designed by the National Marine Fisheries Service Laboratory at Tiburon, California (Nunes and Benville 1978), was constructed (Figure II-l). The "Tiburon" design consisted of a glass tank in which a continuous layer of crude oil was floated over a constant water volume. Seawater was introduced through the top of the solubilizer where it was dispersed in droplet form from a perforated plate above the oil. The droplets then passed through the oil and absorbed the WSF during passage. The WSF solution accumulated at the bottom of the vessel and was discharged through a line to a reservoir. The insoluble oil residue was continually removed through a discharge on the side of the solubilizer.

Use of the "Tiburon" solubilizer with viscous Santa Barbara crude (SBC) oil proved unsuccessful due to the formation of a "mousse". The "mousse" limited the quantity and quality of the WSF that could be obtained and several modifications of the "Tiburon" solubilizer failed to solve problems associated with this formation.

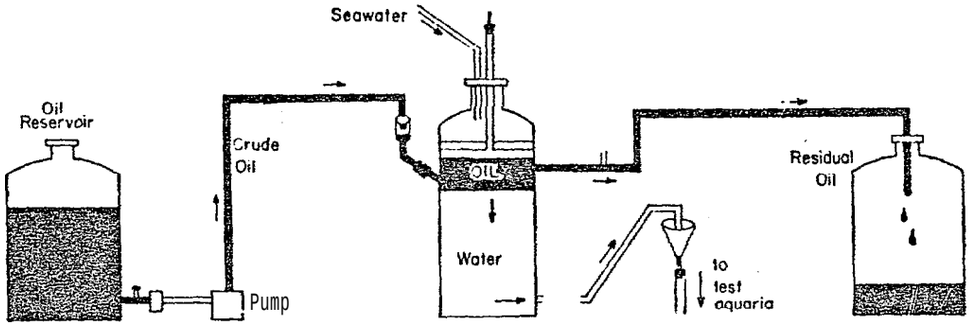


Figure II-1. "Tiburon" solubilizer (Nunes and Benville 1978).

Because of these difficulties, a two-stage solubilizer apparatus (Figure II-2) was designed by MBC (MBC 1981). The first stage provided for the initial mixing of seawater and crude oil, and the second stage separated the WSF from insoluble oil and the "mousse" residue. The solubilizers constructed for embryo/larval experiments and for the adult studies were different in size. This size difference was necessary because of the greater quantity of toxicant required in the adult experiments. As a result of the scale differences, flow rates of oil and seawater were different for each solubilizer.

The embryo and larval experiment flow rates were 0.6 and 0.5 l/rein for oil and seawater, respectively; and for the adult experiment flow rates,

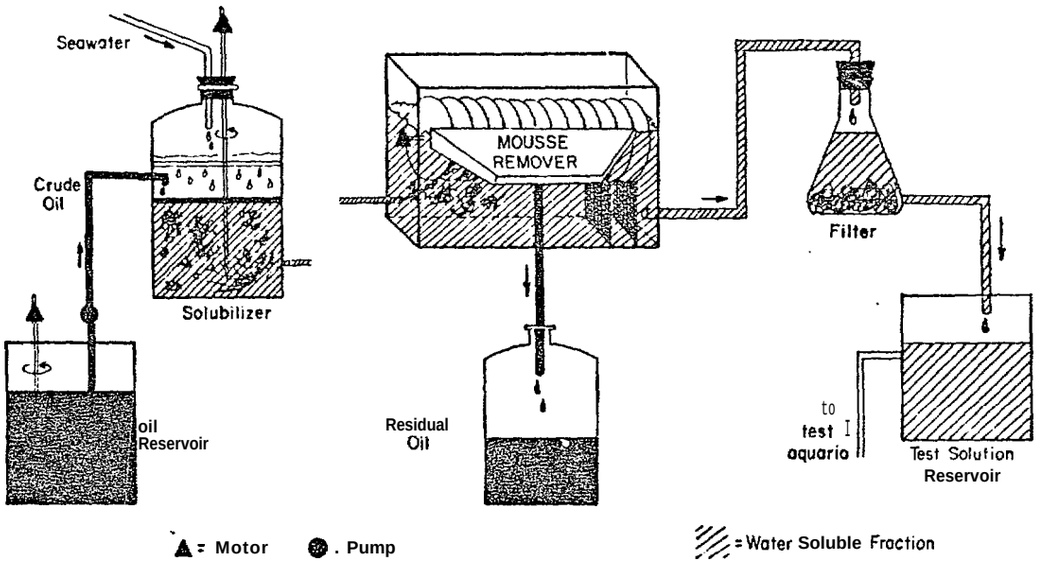


Figure 11-2. MBC crude oil solubilizer for continuous production of water soluble fraction dosing solutions.

3.5 ml/min and 5.5 ml/min, respectively. As in the "Tiburon" design, water was dispersed by a perforated plate positioned above a layer of influent oil. The water droplets percolated through the oil absorbing WSF and simultaneously formed a "mousse". A stirring assembly moving at 4 rpm aided in mixing the oil and water, and promoted accumulation of the "mousse" into globules. The WSF and "mousse" exited through an opening at the bottom of the solubilizer and entered the second stage "mousse" remover.

The "mousse" remover was composed of a rotating bank of discs which moved at a speed of 6 rpm. As the discs passed through the oil-water interface, the "mousse" adhered to the discs. The discs rotated through a squeegee which cleaned them prior to the next revolution. Residual oil flowed by gravity from the combs to a collector barrel while the WSF was passed through a glass wool filter prior to entering a reservoir. WSF solutions obtained from the Santa Barbara crude oil contained approximately 3 to 5 mg/l (ppm) total hydrocarbons. This stock solution was subsequently diluted with seawater to selected experimental dosing levels.

b. Analyses of ?arent Crude Oil and WSF Test Solution

1) Introduction. Santa Barbara crude oil is a complex mixture of predominantly organic constituents. Because of this complexity, analyses were performed to determine the compound specific composition of the crude. Detailed characterizations of the crude were performed each time a new lot was used, each time a bioassay experiment was initiated, and at selected points during bioassay experiments. These characterizations were used not only to elucidate crude oil composition, but also to ensure crude oil uniformity in all experiments. Gas chromatography (GC) and mass spectrometry (MS) were the primary analytical tools employed in these analyses.

The method used to produce WSF test solutions of varying concentration involved continuous generation of a concentrated test solution reservoir

(Section B.1.a., Solubilizer) that was appropriately diluted to achieve the three desired test concentrations. Detailed chemical characterizations were performed on the concentrated reservoir of test solution, as well as each of the four test solutions to which test organisms were exposed. These aqueous test solutions were characterized and monitored over the course of each experiment utilizing a combination of several GC and GC/MS techniques. To completely characterize the petrogenic hydrocarbon content of the WSF solutions, two basic types of analyses were performed. Lower molecular weight volatile constituents were characterized using purge and trap (i.e. direct injection of water into device, purging with carrier gas, then GC reading) techniques, while organic solvent extraction techniques were employed for characterization of intermediate and higher molecular weight constituents.

Test solutions to which organisms were exposed were monitored extensively during all larval and adult experiments. There was no need to monitor the embryo experiments which were of short duration and conducted in sealed containers. The approach utilized in all experiments involved daily monitoring of the volatile portion of the WSF test solution from the control and three exposure regimes (low, medium and high) and weekly characterization of the extractable fraction from each regime. In addition to characterization of the test solution, the daily volatile organic analysis (VOA) was used to monitor exposure levels. Results of these analyses were the basis for adjustment of toxicant-seawater ratios to better achieve target exposure concentrations. Use of VOA as the primary method for monitoring concentrations was considered appropriate because 1) the technique is compound specific and 2) volatile crude oil constituents are generally more water soluble and more toxic than other compunds. Daily VOA samples were analyzed by GC immediately after collection from the influent line to each set of experimental aquaria.

Weekly samples were collected from the control and each exposure regime for the identification and quantification of nonvolatile or extractable components. In conjunction with the corresponding VOA analyses, these analyses provided complete characterization of the type and concentration of toxicants to which test organisms were exposed. A combination of GC and GC/MS techniques were used in characterizing the extractable fraction in weekly test solution samples. The analytical protocol involved analysis of the sample collected from the high concentration exposure level by GC/MS for identification of specific toxicants and elucidation of solution composition. This sample, along with samples from each of the other three regimes, was then analyzed by GC for quantification of individual component concentrations. The GC/MS data were then correlated to GC data for each of the four treatment levels for identification of individual toxicant components. Results of VOA and nonvolatile analyses were summed to determine the total hydrocarbon level to which test organisms were exposed.

2) Analytical Chemistry Techniques. The following sections detail the analytical chemistry techniques used for the characterization of parental crude, WSF test solutions, and tissue hydrocarbon burdens. Where possible, analytical techniques that were common to the characterization of several system components have been grouped together for clarity.

a) Sample Preparation. Where appropriate, the following subsections describe the protocols which were used for collection, preservation and preparation of samples for analysis. The descriptions are grouped according to the type of sample involved, and include crude oils, WSF test solutions and adult organism tissues.

b) Crude Oil Samples. Preparation of Santa Barbara crude oil samples for subsequent analysis entailed solubilization of the crude followed by fractionation into aliphatic, aromatic and polar/heterocyclic components. This method of chemical characterization has been utilized with demonstrated success in analyzing crude oils for hydrocarbon components (Boehm and Fiest 1980, Brown et al. 1980, Payne et al. 1981 a-h).

The separation of aliphatic, aromatic and polar heterocyclic components was accomplished using a liquid column chromatographic fractionation procedure. A 10 mm I.D. x 23 cm long column with a 16 ml pour volume was packed with a hexane slurry of 60/200 mesh activity grade I silica gel. Prior to packing, the silica gel had been cleaned with methylene chloride ($MeCl_2$), activated overnight at 210"C and the cooled in a dessicator. The column was rinsed with 2'0 ml of hexane prior to loading the sample.

The sample was prepared for column chromatography by accurately weighing 50 to 60 mg of the crude oil in a tared vial and dissolving it in 1.0 ml hexane. The hexane/crude solution was then quantitatively transferred to the head of the liquid chromatography column. The column was eluted with solvents of varying polarity to effect the separation into aliphatic, aromatic and polar/heterocyclic constituents. The following solvent elution scheme was utilized for fractionation:

Fraction Type	Elution Solvent	Volume
F1 - Aliphatic	Hexane	30 ml
F2 - Aromatic	Hexane:Benzene (1:1)	45 ml
F3 - Polar/heterocyclic	Methanol :Methylene Chloride (1:1)	60 ml

Each fraction was collected in Kuderna-Danish (KD) concentration apparatus and the solvent volumes were reduced to approximately 1.0 ml on an 80 to 90°C water bath. Fractions F2 and F3 were solvent exchanged to hexane and again reduced in volume to about 1.0 ml each. The concentrated extracts were then transferred to 2.0 ml vials, and when necessary, further reduced in volume at ambient temperature using a gentle stream of purified nitrogen. No extracts were allowed to go to dryness during any extraction or concentration step prior to GC and GC/MS analyses.

c) WSF Test Solution Samples. Analyses were performed on WSF test solution samples for both volatile and non-volatile components. For volatile analyses, samples were collected in 40 ml vials fitted with Teflon-lined septa such that all headspace was excluded. These samples were held at 4°C prior to analysis, which was generally performed within 24 hrs of collection. No sample preparation was required prior to analysis.

The protocol used for analysis of non-volatile hydrocarbons in WSF test solution samples involved solvent extraction with methylene chloride using continuous liquid-liquid techniques. Seawater samples were collected in clean 4.0 liter amber glass bottles and transported to the laboratory on ice. Sample preparation began within 24 hrs of collection to avoid loss and/or degradation of" sample constituents. Each sample was blended in its primary container to obtain a homogeneous mixture, and then 2.0 liters were transferred into a continuous extraction apparatus using a 2.0 liter graduated cylinder. After adding methylene chloride rinses from the graduated cylinder, the pH of each sample was adjusted to 2.0 with 6N H_2SO_4. Each sample was then refluxed in methylene chloride for a 24 hr period to effect extraction of the organic components.

The methylene chloride extracts were then passed through a drying column to remove residual water. The drying columns were prepared by placing a glass wool plug at the bottom of a Pyrex drying column and adding anhydrous sodium sulfate to a depth of 10 cm. The column was rinsed with several portions of methylene chloride, and the sample extract filtered through the drying column into a 500 ml KD concentration apparatus. The flask containing the extract was rinsed three times with 30 ml of methylene chloride and the rinses filtered through the column. The column was then rinsed with a final 30 ml aliquot of clean methylene chloride.

The dried sample extracts were then reduced in volumn using KD apparatus. Boiling chips and a three-ball Snyder column were added to the KD flasks, and the flasks placed in a 60 to 70"C water bath. The vertical position of the apparatus and the water temperature were adjusted as required to complete the concentration in 15 to 20 minutes. When the extract reached an apparent volume of 1.0 ml, the samples were cooled and a two-ball micro Snyder column was attached to the concentrator tube. "The sample volume was then reduced further, and the KD apparatus removed from the water bath and cooled for 10 minutes. The samples were transferred to 2.0 ml crimp top vials and the volume reduced with a gentle stream of purified nitrogen to a final volume of 250 µl.

3) Gas Chromatographic Analysis

a) Analysis of Volatile Components Volatile organic analyses were performed on WSF test solution samples utilizing a Tekmar LSCII purge and trap device together with a Hewlett-Packard 5730 gas chromatography. The procedure employed for these analyses was a modified version of that described by Bellar and Lichtenberg (1974). During the sample concentration step, 25.0 ml aqueous samples were purged for 12 minutes with nitrogen gas at 30.0 ml/minute onto a trap containing Tenax GC polymeric adsorbent. Samples were subsequently backflushed onto a 60°C GC column by Tenax trap resorption at 220°C for four minutes. Following the resorption cycle, the trap material was baked at 250°C for 15 minutes while venting the trap to remove residual organics prior to the next analysis.

An eight foot glass column packed with 1% SP-1000 on 60/80 mesh Carbopack B was used for separation of the volatile petroleum hydrocarbons. GC operating parameters for these analyses were as follows:

detector - flame ionization
detector temperature - 200°C
injector temperature - 250°C
oven temperature - 60°C to 220°C at 8°C min with a 2 min initial hold and a 30 min final hold
carrier gas - 40 ml/min nitrogen at 60 psi

All samples were quantified against a calibration standard prepared by spiking seawater samples with a methanol solution containing a known amount of selected petroleum hydrocarbons. Nine volatile aromatic and aliphatic hydrocarbons encompassing the range of 5 to 9 carbon atoms were used for the calibration standard. These hydrocarbons included n-pentane, n-hexane, cyclohexane, benzene, toluene, p-xylene, isooctane, cumene and n-nonane. Initial GC/MS studies of the seawater soluble fraction indicated that these volatile compounds were present in the greatest abundance. As a result of these studies, a standard composed of these compounds was considered most appropriate for instrument calibration and sample quantification.

At least one calibration standard was analyzed with each set of WSF test solution samples from bioassay experiments. From these analyses, response factors were generated for each of the nine standard components. Concentration levels for each sample component were then determined using the response factor generated for the corresponding standard constituent. Any sample component which did not correspond to a standard constituent was quantified using the response factor of the closest eluting standard component. Seawater blanks were also analyzed with each sample set to evaluate the level of background interference.

b) Analysis of Non-volatile Components. All gas chromatographic analyses for non-volatile components (WSF test solution and tissue samples) were performed on a Hewlett-Packard 5840A gas chromatography equipped with a 18835A glass capillary inlet system and flame ionization detector. The microprocessor-based instrument was interfaced to a Texas Instruments Silent 700 data terminal equipped with cassette tape drive for direct storage of calibration data, oil sample component retention times, and peak areas required for data reduction.

A 30 m J&W Scientific Company SE-54, wall-coated, open tubular glass capillary column was utilized for the desired chromatographic separation of hydrocarbon compounds. The following temperature programming was used for all analyses:

Initial temperature: 50°C for 5 min
Program rate: 3.5°C/min
Final temperature: 275°C for 20 min

The injection port and detector were maintained at 270 and 350°C, respectively. All injections were made in the splitless mode of operation with an injection port backflush at 1 min into the run. Constant injection volumes of 2.0 µl were analyzed using a Hewlett-Packard 7671A automatic liquid sampler, which increased precision substantially relative to manual injections. Hydrocarbon standards were analyzed to provide tentative identifications of sample components prior to GC/MS analyses.

c) Gas Chromatogram Data Reduction. Hydrocarbon concentrations of individual resolved peaks in each sample chromatogram were calculated by computer using the formula given in Equation 1. This is an example of the program used for crude oil analyses, and calculations for aqueous or tissue samples were very similar.

g compound X/g crude oil =

$$(Ax) \times \left(R.F.\right) \times \frac{P.I.V._{.1}}{Inj.\ S.\ Vol.} \times \frac{Pre\text{-}C.S.Vol.}{Post\text{-}C.S.Vol.} \times \frac{100}{\%NSL\ on\ LC} \times \frac{100}{\%\ DW/FW} \times \frac{1}{g_1} \quad (1)$$

where:

Ax = the area of peak X as integrated by the gas chromatograph (in arbitrary GC area units)

R.F. = the response factor (in units of µg/GC area unit)

P.I.V. + 1 = the post injection volume (in µl) from which a one µl aliquot had been removed for analysis by GC (measured by syringe immediately following sample injection)

Inj. S.Vol. = the volume of sample injected into the GC (always 1.0 µl as measured by the automatic liquid sampler)

$\frac{\text{Pre-C. S. Vol.}}{\text{Post-C.S.Vol.}}$ = the total solvent volumes before and after an aliquot is removed for gravimetricanalysis on a Cahn elect ro-balance

%NSL on LC = the percent of sample non-saponifiable lipid used for SiO_2 column chromatography

%DW/FW = the percent dry weight of wet weight in the oil sample being analyzed

g = grams wet weight of oil

During analysis of the extracts, the gas chromatograph was recalibrated after every 8 to 10 injections and individual response factors were calculated for all detected even and odd n-alkanes between nC_8 and nC_{32}. By incorporating the post injection volume (PIV) into the calculation, the amount of hydrocarbons measured in the injected sample was converted to the total hydrocarbon concentration in the sample.

Assignment of KOVAT indices to branched and cyclic compounds eluting between the n-alkanes was done by interpolation using the unknown compound and adjacent n-alkane retention times. Concentrations of these components (branched and cyclic) were calculated by linear interpolation of the adjacent n-alkane response factors and the unknown compound peak's KOVAT index. Assignment of KOVAT indices to peaks in the aromatic fraction was made by direct correlation of unknown peaks with retention times from the n-alkane-standard run completed prior to sample injection {Payne et al. 1978). Further confirmatory analyses were performed by GC/MS.

Unresolved complex mixtures (UCM's) were measured in triplicate by planimetry; the planimeter area is converted to the gas chromatograph's standard area units at a given attenuation and then quantified using the average response factors of all the n-alkanes occurring within the range of the UCM, as shown in Equation 2.

$$\frac{g\ UCM}{liter} = Area_p \times (Conv.\ F) \times \frac{S.Att.}{Ref.Att.} \times (R.F._{a,b}) \times [\ldots] \qquad (2)$$

where:

$Area_p$ = UCM area in arbitrary planimeter units

Conv. F. = a factor for convert ing arbitrary planimeter units to GC area units at a specific GC attenuation

$\frac{\text{S. Att.}}{\text{Ref. Att.}}$ = the GC attentuation at which the sample chromatogram was run and the reference attenuation to determine the conversion factor (Conv. F.), respectively

$R.F._{a,b}$ = the mean response factor for all sequential n-alkanes (with carbon numbers a to b) whose retention times fall within the retention time window of the UCM

[...] = the same parameter: enclosed in brackets in Equation 1

The GC was recalibrated daily or after every 8 to 10 injections with n-alkane standards. Response factors and retention times of the n-alkane standards were tabulated daily and checked against previous analyses. Corrective action was taken if the response factors and retention time of the standards were found to deviate from the expected values. Instrument parameters were checked daily to insure that the GC and supporting hardware were functioning properly. Standard mixtures were analyzed with each set of test solutions to aid in compound identification in sample chromatograms. The

standards analyzed included lower and higher molecular weightaromaticcompounds, phenol and alkyl-substituted phenols, n-alkanes, and alkyl-substituted alkanes. Tentative identifications of sample components were made by comparing sample chromatograms with standard chromatograms, and confirmation of the tentative identifications was performed by GC/MS analysis.

4) Gas Chromatographic (GC)/Mass Spectrometric (MS) Analyses. GC/MS analyses were performed on a Finnigan 4021 quadrupole gas chromatograph/mass spectrometer. This instrument is equipped with an INCOS data system which provides GC/MS data system functions and allows comparison of selected mass spectra against a library of more than 38,000 entries by sophisticated pattern recognition techniques. All data acquired on the GC/MS instrument were stored on a magnetic tape.

A 30.0 meter J&W SE-54 glass capillary column (0.25 mm I.D. with a film thickness of 25 μm) was used to achieve chromatographic separation, and the injection port configuration was operated in the splitless (Grob/type) mode. The statictime upon injection was 1.0 rein, after which time the injector was backflushed with the split and septum sweep flows at a combined rate of 35.0 ml/min. The GC oven temperature was programmed to remain isothermal at 50°C for 5.0 min following injection, then programmed to 275°C at 3.5*C per min and held at 275°C isothermally for approximately 20 min.

The column effluent from the capillary system of the GC was directly transferred into the ion source which was operated in the electron impact mode. The ion source was operated at 70 eV and the lens potentials optimized for maximum ion transmission. The quadruple offset and offset programs were adjusted to yield a fragmentation ratio of perfluorotributylamine m/e (69/219) of 2.5:1. This tuning yields quadupole electron impact spectra that are comparable to magnetic sector electron impact spectra, thereby allowing optimal matches in the computer search routines used in the INCOS and other spectral search systems. The mass spectrometer was controlled by an INCOS data system which scans the quadruple rods from 35 to 500 amu in 0.95 sec. A hold time of 0.05 sec between scans allows the electronics to stabilize prior to the next scan. The mass spectrometer was tuned at the beginning of each day using perfluorotributylamine. Calibration was accomplished with a routine diagnostic fit of 2% mass accuracy. Prior to analysis of samples, a standard mixture of hydrocarbons was injected and analyzed.

The identification of components in the test solutions was accomplished by GC/MS computer library searches using the National Bureau of Standards library of mass spectra for GC/MS analyses. Numerical fit indices were calculated for tentative compound identifications in sample extracts. A measure of the similarity of the background-subtracted experimental mass spectrum and the reference spectrum was determined as follows:

$$\text{Purity} = 1000\left[\Sigma E(m) \times R(m)^2\right] / \left(\left[\Sigma E(m) \times E(m)\right] \times \left[\Sigma R(m) \times R(m)\right]\right),$$

where E(m) and R(m) are the square roots of the mass weighedintensities (above 1% relative abundance) in the experimental and reference spectra respectively. In addition, a measure of the degree to which the reference spectrum is contained in the background subtracted experimental spectrum was determined as follows:

$$\text{Fit} = 1000\left[\Sigma E'(m) \times R(m)^2\right] / \left(\left[\Sigma E'(m) \times E'(m)\right] \times \left[\Sigma R(m) \times R(m)\right]\right),$$

where E'(m) is for all masses (m) common to both the reference and experimental mass spectra. Compounds were positively identified in test solutions when there was a high degree of correlation between sample and standard spectra as determined by these criteria.

2. TISSUE ANALYSIS

a. Analyses of Tissue Hydrocarbon Burden

1) Radioactive Tracer. A ^{14}C labeled naphthalene tracer was used as a surrogate for monitoring petrogenic hydrocarbon uptake in all embryo and larval experiments. Use of a radiolabeled tracer was necessitated by the analytical limitations associated with characterizing tissue burdens from organisms with very little biomass. Direct characterization of tissue hydrocarbon burdens with adequate sensitivity would have required a biomass many times greater than that available in these experiments. The naphthalene tracer was selected because: 1) naphthalene was a major component of the parental Santa Barbara crude oil; 2) it appears to be accumulated more and retained longer than other hydrocarbons (Neff et al. 1976); and 3) it was readily available from vendors and did not require custom synthesis.

a) Test Concentration Tracer Levels. A stock solution of aqueous ^{14}C-naphthalene was prepared for each WSF concentration. Tracer stock solutions were stored 'in '4.0 liter amber glass bottles and delivered to the appropriate test concentration at 0.3 ml/min using a multistatic pump system. The tracer was mixed with the test solution in a mixing column prior to entering the test tanks.

Specific activity levels of the three WSF test concentrations were monitored weekly. The specific activity was determined by adding 0.5 ml of test solution to 10.0 ml of liquid scintillation cocktail (Beckman Ready-SoIv) and determining the decays per minute (dpm) on a Beckman LS 100C liquid scintillation counter. Target values for specific activity in the low, medium, and high concentrations were 3, 30, and 300 (dpm). The tenfold increase in tracer levels between concentrations related to the differences established for hydrocarbon content in the three concentrations (i.e. 5, 50, and 500 ppb). The concentration of ^{14}C naphthalene tracer added to each WSF test solution represented a minute percentage of the total naphthalene present at each concentration. This approach was used to ensure that addition of the tracer would not change the characteristics of the WSF test solution.

b) Tracer Tissue Level Determination. ^{14}C-naphthalene incorporated into embryo and larval tissues was analyzed using liquid scintillation procedures: The embryos or larvae were washed in clean seawater and filtered onto a Millipore HA membrane filter. The filter and 0.5 ml of Beckman TS450 tissue solubilizer were placed in a liquid scintillation vial for 48 hrs. Ten milliliters of scintillation cocktail were added following the 48 hr period. This mixture resulted in a white cloudy liquid which was allowed to clear prior to placing the vial in the liquid scintillation counter.

2) Tissue Extracts. Selected tissues were directly analyzed for petrogenic hydrocarbon content using GC and GC/MS techniques in all adult experiments. Target tissues were analyzed from background, control and exposed (three exposure concentrations) test organisms in each experiment (Figure II-3). Background analyses were performed on organisms which were collected at the same time as those used in the bioassay experiments. Results

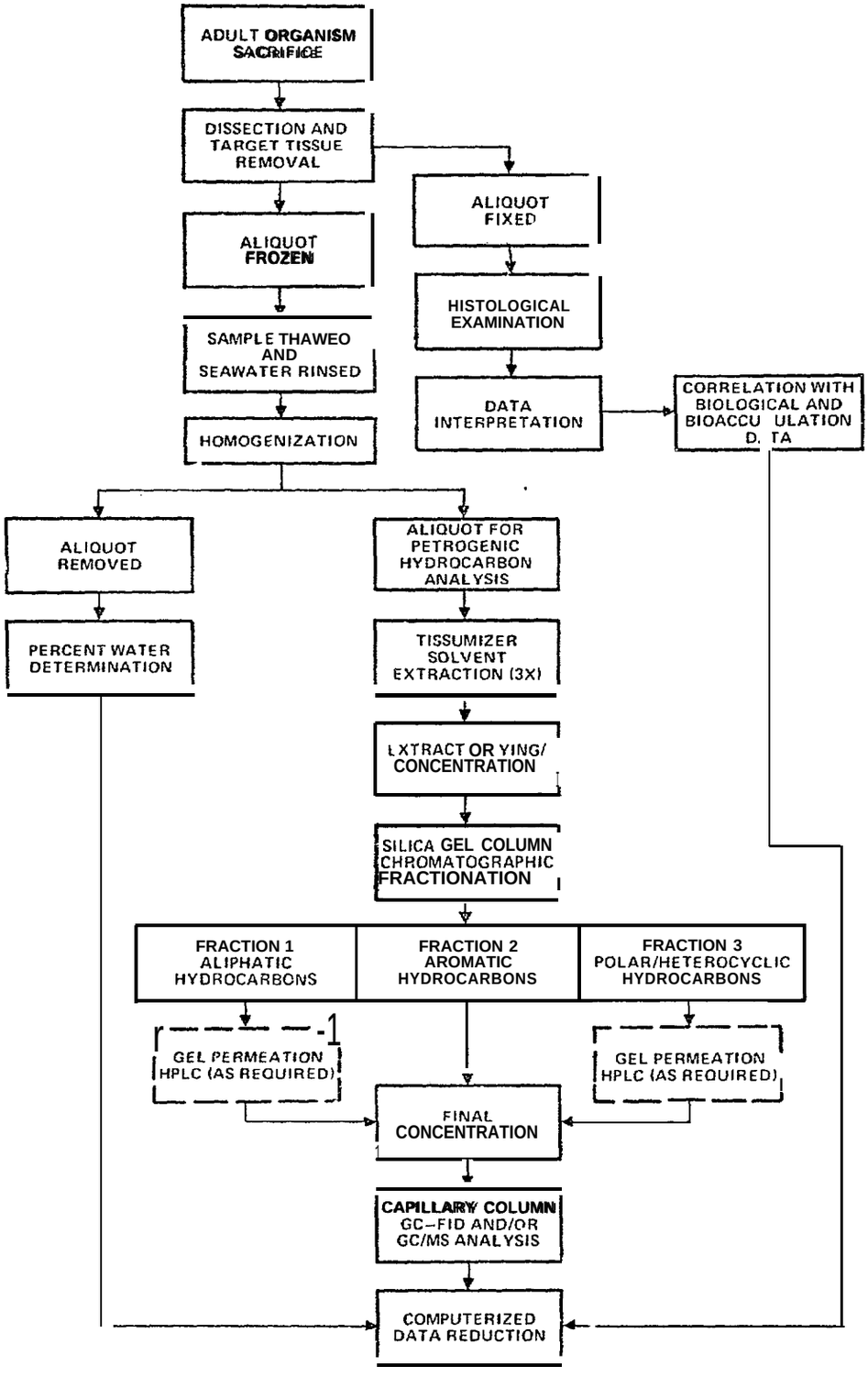


Figure II-3. Flow diagram for hydrocarbon sample preparation and analysis of tissue

of these analyses were compared with those of control organisms to evaluate potential contamination of control organisms during each experiment.

Triplicate tissue samples were analyzed in all cases where sufficient biomass was available. The general analytical approach entailed complete characterization of one of the three replicates by GC/MS, and complete GC analysis of the remaining replicates. The GC/MS data were used for component identification, with all quantification performed by GC. The qualitative GC/MS data from one of the three replicates were correlated with the remaining two replicates for identification of individual compounds.

a) Adult Tissue Samples. Adult organisms were dissected and the target tissues removed for chemical analysis. The dissected tissue samples were placed in chemically cleaned Pyrex containers and frozen prior to shipment. Samples remained frozen during shipment and upon receipt at the laboratory until analysis. Immediately prior to preparation, tissue samples were thawed at room temperature and aliquots separated for dry weight determination and hydrocarbon analysis.

Triplicate 1.0 to 2.0 g aliquots were placed in Pyrex beakers, homogenized and transferred to tared aluminum weighing boats. The homogenized samples were then weighed and placed in a 95°C convection oven for 3 to 5 days to determine percent moisture. The samples were removed, dessicated and reweighed periodically until a constant dry weight was obtained.

The procedure utilized for tissue extraction is a modified version of the protocol described by McLeod et al. (1981). Figure II-3 summarizes the analytical protocol employed for these analyses. For each replicate, a 5.0 to 20.0 g aliquot of wet tissue (dependent upon biomass available) was placed into a tared aluminum weighing boat and the wet weight determined. The sample was then transferred to a rectangular Pyrex beaker, and the tissue thoroughly homogenized with a Brinkman Polytron ultrasonic/ mechanical Tissumizer. A 75.0 ml portion of methylene chloride and 10.0 g of anhydrous sodium sulfate were added to the homogenized tissue. This mixture was extracted for three minutes using the Tissumizer. The mixture was then allowed to settle and the methylene chloride layer was removed and passed through a Pyrex funnel fitted with solvent extracted filter paper and anhydrous sodium sulfate.

The effluent from the Pyrex funnel was in turn passed through a column of anhydrous sodium sulfate and collected in a KD flask fitted with a 10.0 ml concentrator tube. This process was repeated two more times with clean 75.0 ml aliquots of methylene chloride. On the final (third) extraction, the entire contents of the extraction vessel were decanted into the Pyrex funnel and rinsed with clean methylene chloride to assure that quantitative transfer was achieved. Spike/recovery studies indicated that this extraction technique resulted in excellent recovery of even low levels of petroleum hydrocarbon species and eliminated residual water which adversely affected subsequent preparative procedures.

The combined methylene chloride extracts were then reduced in volume to approximately 1.0 ml using KD apparatus and solvent exchanged to hexane as described in the subsection on aqueous test solution sample preparation. The solvent exchanged extracts were fractionated into aliphatic, aromatic, and polar/heterocyclic components using the protocol described

in the crude oil preparation subsection. The fractionated extracts were reduced in volume to approximately 0.5 ml. At this point all F2 [aromatic) extracts were transferred to appropriate vials and further concentrated to a final volume of 100 µl.

All F3 (polar) extracts and selected F1 (aliphatic) extracts were subjected to gel permeation high pressure liquid chromatography to remove very large biogenic components such as cholesterol and large fatty acid esters. F3 extracts and selected F1 extracts were so heavily laden with these constituents that further cleanup was required to obtain appropriate concentration factors and meaningful results. Spike/recovery studies indicated that the gel permeation chromatographic technique was effective in removing these large biogenic components without adversely affecting recovery of petrogenic hydrocarbons. Following cleanup by gel permeation chromatography, all extracts were concentrated to a final volume of 100 µl for analysis.

b. Histopathology Methods

1) Embryo and Larvae. Exposed and control embryos and larvae of each target species were fixed for several days in phosphate buffered formalin (PBF) or formol acetic acid (FAA) consisting of 10 parts formaldehyde, 90 parts 70% ethanol, and 5 parts glacial acetic acid. FAA proved to be the best fixative, and also served as a decalcifying agent for larval tissue.

Whole embryos and larvae of each test species were examined for gross abnormalities using a dissection microscope and photographed at 5 to 20X using Zeiss photographic equipment. A metric scale was placed in the photographic field to facilitate size comparisons between experimental and control organisms. Following gross observation and photography, subsamples of eggs and larvae were embedded in paraffin, sectioned at 6 to 8 microns and stained with hematoxylin and eosin. Mounted tissue sections were examined using a Zeiss bright field microscope equipped with planapochromatic lenses.

Standard and developmental abnormalities were most easily seen in cleared, whole specimens of embryonated eggs and larvae. Histological sections were less useful since gross abnormalities were less easily appreciated, and cellular changes in developing organs were difficult to detect and interpret. Accordingly, major reliance was placed upon examination of whole embryos and larvae for developmental failures and anomalies. Histological sections of embryos and larvae were nevertheless examined in the event that cellular changes such as neoplastic transformation might be observed. Abnormal cellular changes that were evaluated for various organ tissues are listed in Table II-3. Terms are defined in the report glossary.

2) Adults. The six target tissues for histopathological evaluation of northern anchovy and California halibut included gill, liver, gut (mid-intestine), gonad, skeletal muscle, and eye. Those for the California mussel included gill, gonad, digestive gland, and muscle (pedal retractors and abductors). Tissues were fixed in PBF or FAA for several days followed by standard histological preparation and hematoxylin and eosin staining. A few tissue sections were also stained with periodic acid Schiff (PAS) reagent to detect liver cell glycogen. interpretive criteria used for the evaluation of each target tissue are described in Table II-3.

Table II-3. Target organ tissues evaluated for abnormal cellular conditions which may result from toxicant exposure.

	Target Organ Tissue							
	Gill[2,3]	Gonad[2,3]	Liver[2]	Gut[2]	Digestive[3] Gland	Muscle[2] Skeletal	Muscle[3] Retractor/Adductor	Eye
Abnormal Cellular Condition Examined[1]	•							
Edema	•							
Necrosis	*	•	•	•	*	*	•	
Hyperplasia	•							
Hypertrophy	•							
Fibrous Obliteration	•							
Microaneurisms	•							
Glycogen Depletion			*					
Fat Vacuolation			•					
Hyaline Acidophil Bodies			•					
Fibrosis			•					
Regenerative Nodules			*					
Melanin-Macrophage Centers			*					
Preneoplastic and Neoplastic Changes			*					
Pyknosis				*				
Exfoliation of Epithilium				*				
Intestinal Inflammation				*				
Parasitism		*						
Hypoplasia						*		*
Neuritis/or Neural Degeneration								•
Corneal Edema								*
Cataract Formation								*
Inflammation/Degeneration of Retina				•				
Acidophilic Granular Amoebocytes				•				
Basophilic Granular Amoebocytes				•				
Brown Pigmented Cells								

1 condition defined in Glossary
2 in California halibut
3 in California mussel

3. CALIFORNIA HALIBUT (Paralichthys californicus)

a. Embryo Experiment

1) Collection of Test Organisms. Male and female Paralichthys californicus in ripe and running condition were captured by ottertrawl at a depth of approximately 16 m offshore from the entrance to Anaheim Bay in Long Beach, California. In the laboratory, eggs were hand-stripped and artificially fertilized. Fertilized eggs were washed of excess sperm and observed for 1 to 2 hours to ensure that initial cleavage had occurred. The embryo exposure experiment was conducted using these early stage embryos.

2) Experimental System and Protocol. Embryos of P. californicus were continuously exposed to three water soluble fraction (WSF) test solutions (low, medium and high concentrations corresponding to approximately 5, 50 and 500 ppb) and a seawater control. One hundred developing embryos were placed in each of six 900 ml replicate containers for each test solution concentration and the control. The experiment was conducted under static conditions following an initial dosing of embryos with the appropriate test solution concentration. Each replicate container was completely filled with test solution and tnen sealed with a screw cap and Teflon tape to prevent loss of the volatile test solution components. Before introduction of embryos, 0.6 ml of radiolabeled tracer was added to each replicate at the appropriate concentration to determine hydrocarbon uptake by embryos. The experiment was conducted under indirect light conditions (a 12 hr L: 12 hr D cycle) at a temperature of 16.0°C.

The embryo experiment was initiated approximately 1 to 2 hours after fertilization and allowed to continue until hatching was completed. Upon termination of the experiment, the numbers of (a) dead and live embryos and (b) dead, live and malformed newly-hatched larvae were counted in each

replicate to determine embryo mortality and hatching success. In addition, the size at hatching was determined by measuring 10 randomly selected larvae from each replicate. A maximum of 25 larvae was obtained from each replicate for the assessment of tracer uptake while all remaining larvae were fixed in 4% formalin-seawater for histological examination.

b. Larval Experiment

1) Collection of Test Organisms. Fertilized P. californicus eggs were obtained in a manner identical to that used in the embryo experiment. Developing embryos were held in aerated aquaria at 16°C for approximately 36 hrsprior to use in the larval experiment.

2) Experimental System. Larval P. californicus were exposed to three water soluble fraction (WSF) test solution regimes (low, medium and high concentrations) and a seawater control under flow-through conditions. Test chambers used in the larval experiment were of 3.8 liter glass jars with 3.0 cm openings bored in their bases. The mouth of each was covered with 41 micron mesh nitex screen which allowed for contiguous test solution circulation, yet minimized larval loss. Screens were changed every three days to control fouling and flow reduction. Each jar was inverted in an 18.0 liter aquarium and supported by glass rods to facilitate flow through the jar. WSF test solution was delivered through the 3.0 cm opening, with constant level siphons maintaining the volume of test solution in each jar and holding aquarium. A total of 12 replicates, was used for each test solution concentration and control.

3) Experimental Protocol. Halibut larvae were continuously exposed to three WSF test solutions (low, medium and high concentrations) and a seawater control under flow-through conditions. The experiment was initiated by placing 150 fully developed embryos in each replicate test chamber just prior to hatching.

To maintain continuous exposure and yet examine larval responses to WSF exposure during critical developmental stages, 12 replicate chambers were used for each WSF concentration and the control. Three replicates were randomly sacrificed from each WSF concentration and the control on exposure day 3 after daily observations indicated that no larvae were visible in the water column of the high concentration. The second group of three replicates was terminated on larval exposure day 7 just prior to the period when non-feeding larvae would die of starvation. The third group of replicates (only control and low concentration larvae remained at this time) was terminated on larval exposure day 14 while the remaining group of the three replicates in the low concentration and control was terminated on day 18 of the experiment following a noticeable decline in the larval density in the low concentration chambers.

Upon termination of individual replicates, larval mortality, growth, radiotracer uptake and development were assessed and samples were obtained for histopathology. A flow diagram illustrating the experimental protocol employed in the larval P. californicus experiment is presented in Figure II-4.

4) Larval Feeding. The rotifer Brachionus plicatilis was used as food for P. californicus larvae. Rotifers were presented to the larvae on day 3 just prior to yolk sac absorption. When food was delivered, it was necessary to discontinue the flow of test solution to the experimental

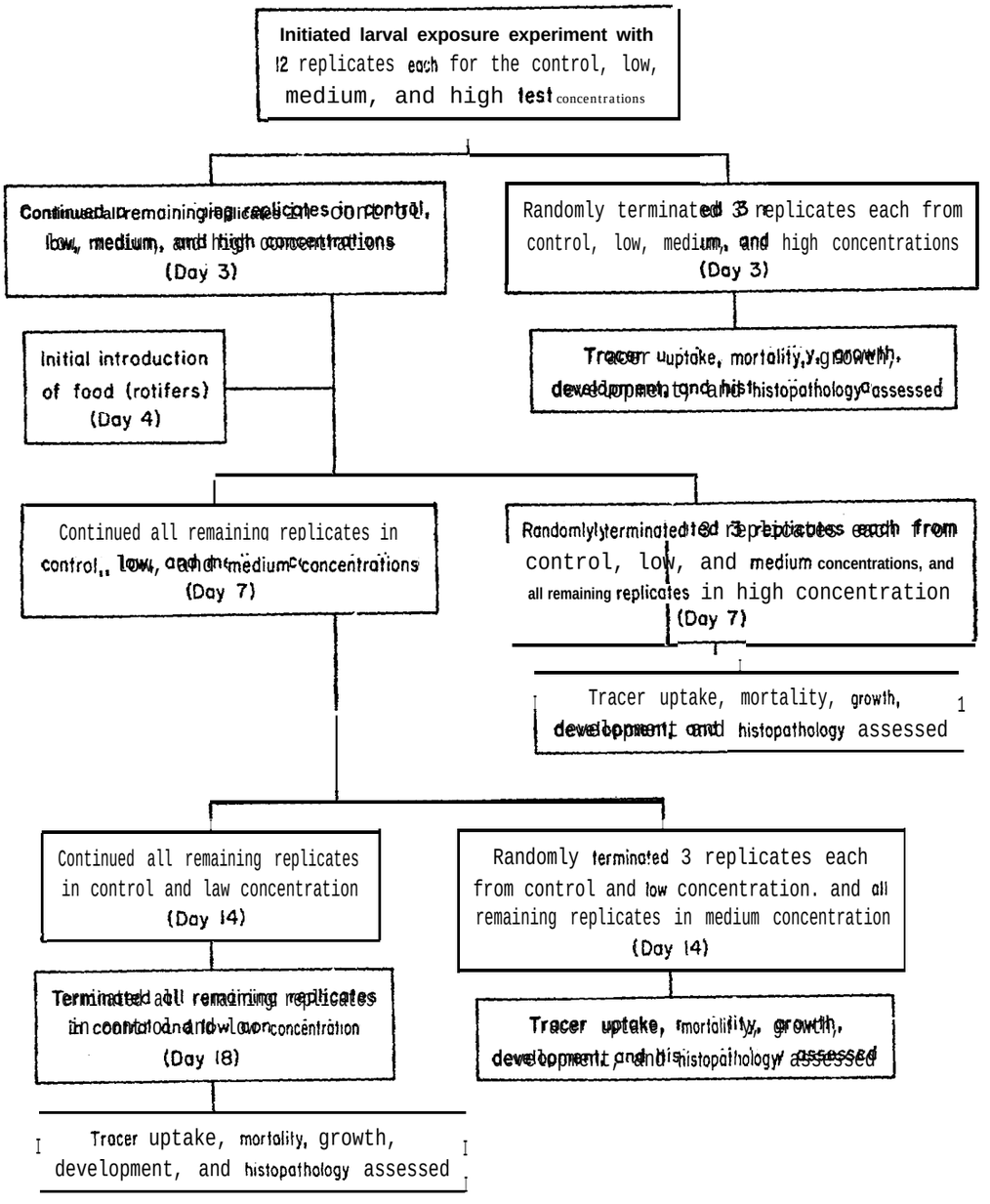


Figure II-4. Flow diagram depicting experimental protocol employed in larval Paralichthys californicus exposure experiments.

chambers to reduce disturbance of the larvae. To prepare rotifers for inoculation, a volume of the source rotifer culture sufficient to produce a final density of 30 to 50 rotifers/ml in each aquarium was screened through 20 micron mesh nitex screen. The retained rotifers were washed into a glass

beaker with the appropriate test solution concentrations and then placed into the larval feeders. Rotifers were dispensed through Tygon tubing into the culture jar at a rate of 5 to 10 ml/min. After dispensing, the rotifer density in each chamber was checked to ensure that the desired range had been achieved.

The food concentration in each WSF concentration was checked daily in two randomly selected chambers. Density estimates were based on the mean of five 1.0 ml subsamples from each chamber. When the mean density fell below 30 rotifers/ml the experimental aquaria were flushed out and a new rotifer culture added.

Experimental chambers were flushed after replacing the 41 micron screen with 202 micron mesh nitex screen. The 202 micron mesh was left in place for 1 to 2 hrs while the system was thoroughly flushed. Following flushing, a clean 41 micron mesh screen was placed on the mouth of the culture jar and a new rotifer inoculation added.

5) Daily Monitoring and Observations. Dissolved oxygen, pH, temperature, salinity and concentration of each test solution were measured daily. Water quality parameters were measured in randomly selected chambers from each WSF concentration and the seawater control. This procedure was established after several weeks of testing indicated that there was no detectable difference in the water quality parameters between replicate chambers.

To aid the daily visual observation of larvae, three sides of each experimental chamber were darkened, while one side was left clear. Even with the dark background, it was not possible to make in situ observations of larvae less than 4.0 mm (approximately 2 to 3 days old). To maintain visual surveillance of larvae less than 3 days old, three static replicates were established for each WSF concentration. Static chambers consisted of 3.8 liter screw top glass jars containing the appropriate test solution. Twenty-five larvae were placed in each jar and the lid tightened and sealed with Teflon tape. Daily observations were made on the larvae in each static jar utilizing fluorescent backlighting. Observations made from both the static and flow-through test chambers included: 1) location of larvae within the water column; 2) mobility of larvae; 3) horizontal or vertical orientation of the larvae within the water column; and 4) an estimate of the number of live larvae in each aquarium.

c. Adult Experiment

1) Collection of Test Organisms. Adult California halibut were collected offshore from Newport Beach, Huntington Beach. and Sunset Beach. California. Collections were made by otter trawl-in late November and early December 1981. Fish were returned to the laboratory and held for approximately 1 week until capture-associated mortality had stabilized. Following laboratory acclimation, fish were placed in 300 gallon tanks and transported to Santa Cruz, California on three separate occasions by flatbed truck. All trips to Santa Cruz were made at night to reduce thermal stress and required approximately 8 hrs.

2) Experimental System. The experimental system for adult halibut had an open flow-through configuration. Such a design provided the best alternative for approximating realistic environmental conditions (pH, dissolved oxygen, temperature, etc.) in the laboratory. Source water for the adult experiment

was pumped from Monterey Bay, sand-filtered (removing 5 to 45 micron sized particulate) and stored in twin fiberglass 132,500 liter (35,000 gallons) silo storage tanks.

Experiments were conducted in 1,350 liter (360 gallon) circular fiberglass aquaria measuring 152.5 cm (60 inches) in diameter and 76.2 cm (30 inches) in depth. Aquaria were covered with fiberglass tops having two acrylic windows that admitted light. The tops also provided shade, served as dust covers, and minimized fish stress by reducing disturbances.

Seawater from storage tanks flowed directly into the experimental tanks and into the solubilizer system. A separate system supplied WSF toxicant to the aquaria (Section B.1.a, Solubilizer). Each aquarium was equipped with two ball-type flowmeters permitting independent control of seawater and toxicant flow. The relative proportion of seawater and toxicant was adjusted to achieve the desired concentration in each tank. Control tanks received only filtered seawater and were equipped with a single valve to regulate flow. The total flow rate into all experimental aquaria (seawater or seawater plus WSF) was maintained at approximate 4 l/rein (1 gal/rein), equivalent to a turnover rate of approximately five times per day. Water levels in the tanks were maintained with a two-piece stand-pipe assembly which helped control volatile loss and maintained test concentrations by drawing water from the tank bottom.

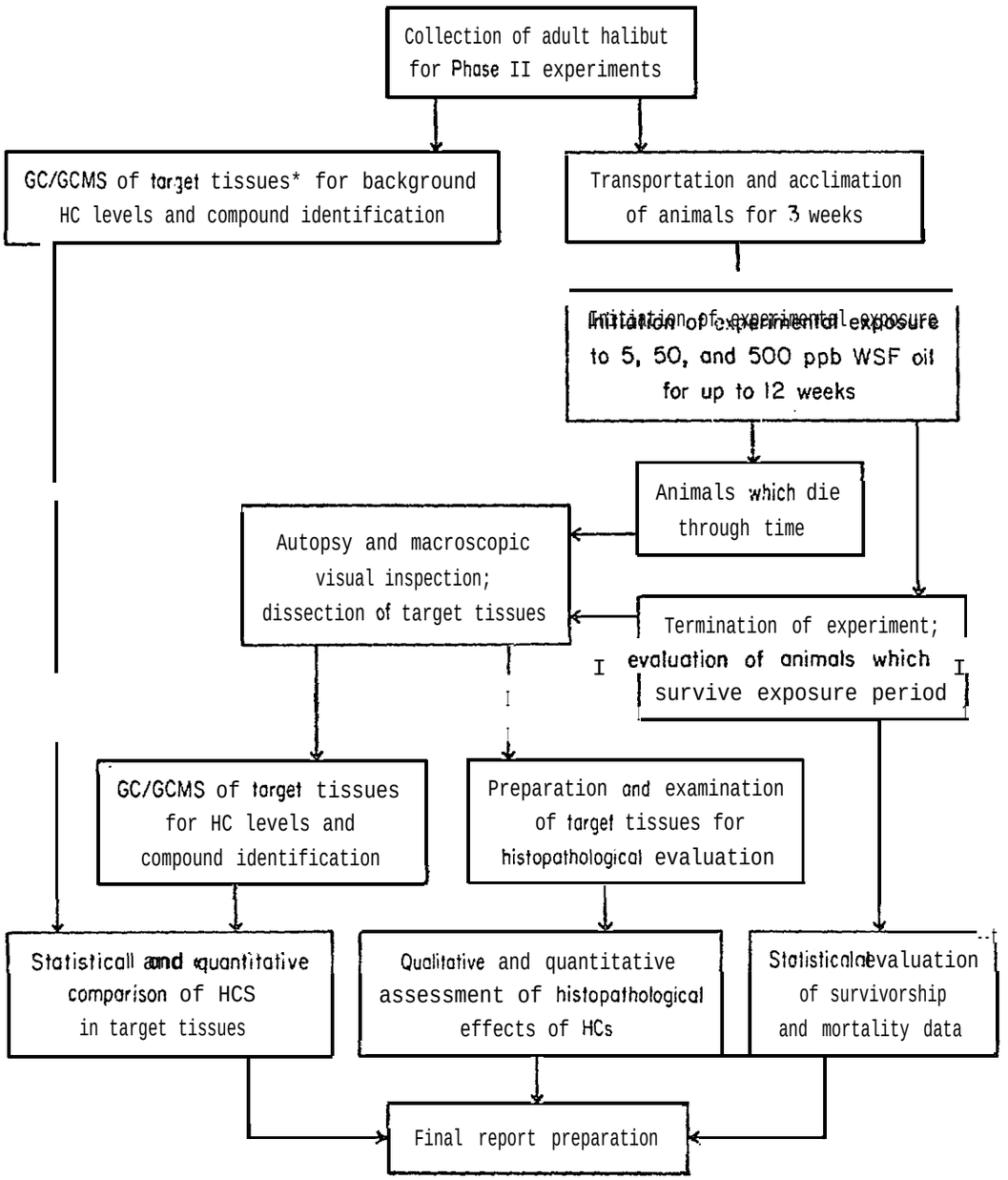
Effluent water from the experimental system contained hydrocarbons that needed to be removed prior to discharge. Hydrocarbon removal was accomplished by passing the effluent through two successive filtration systems. The first filtration system contained a bank of five activated charcoal filters (15 kg/filter). The second system contained nine open-celled foam filters arranged in series (Dr. Brock DeLappe, University of California Bodega Marine Laboratory, personal communication). Samples of effluent were regularly analyzed for total hydrocarbon content.

3) Experimental Protocol. Adult fish were transferred to a holding tank and acclimated for four weeks upon arrival at the Santa Cruz facility. The acclimation period allowed for initial mortality and permitted recovery from stress of capture, handling, and transport. During acclimation animals were examined for signs of disease, parasites, or abnormalities which might preclude their use in the long-term experiment.

Background hydrocarbon levels were measured in gill, gonad, digestive tract, eye, liver and muscle tissue prior to beginning the experiment (Figure II-5). All test animals were individually tagged, measured to standard length, weighed, and randomly assigned to experimental tanks. Five halibut were placed in each of three replicate tanks for a total of 15 individuals per exposure regime.

The adult halibut experiment was initiated on January 17, 1982 and was originally scheduled to continue for a 120-day period. As test fish died during the experiment they were measured, weighed and then frozen for subsequent removal of target tissue samples for analysis of hydrocarbon tissue burden and histopathological evaluation. After 80 days of exposure, the experiment was terminated and all remaining live animals were sacrificed and processed in a similar manner.

4) Daily Monitoring. Monitoring activities included: 1) behavioral observations, 2) removal and processing of fish that died, 3) qualitative



*Muscle, liver, gonad, digestive tract, eye, and gill

Figure II-5. Flow diagram depicting experimental protocol employed in adult Paralichthys californicus exposure experiments.

observations related to the overall health of test fish, 4) feeding and determination of food consumption by test fish, 5) water quality measurements, 6) general aquarium maintenance, and 7) WSF test solution concentration monitoring in experimental and control aquaria, with adjustments as required.

The general condition of test fish was evaluated by making observations on mortality, behavioral changes, and the presence of abnormalities and disease symptoms. Evidence of parasites, scale loss, coloration changes, abrasions, and abnormal behavior were recorded daily.

General maintenance of fish included feeding, tank cleaning, and medication. These activities were performed routinely for each species. In addition, operation of equipment and the overall system was checked daily and repaired as necessary.

Water quality was evaluated daily by measuring salinity, pH, temperature, and dissolved oxygen. In addition, WSF test solution concentrations were monitored daily by measuring the volatiles in experimental and control tanks using GC analysis (Section B.1.b.2.), Aqueous Test Solutions). The extractable component was sampled and sent to Science Applications, Inc. in San Diego, California, for weekly analysis. The total hydrocarbon exposure concentration was considered to be the sum of the WSF and extractable components.

Tissue samples were obtained from each test organism following termination of the experiment and preserved in 10% formalin for subsequent histological processing and evaluation. Replicate composited tissue samples were also obtained from fish in each NSF test solution concentration and control for subsequent hydrocarbon tissue burden analysis.

5) Feeding. Adult fish were fed a daily ration of 60.0+10.0 g of live Callianassa or frozen squid or anchovy depending on food-avail-ability. The quantity of food consumed was monitored by record"ing the quantity fed and subtracting the weighed remnants after 24 hours.

4. NORTHERN ANCHOVY (Engraulis mordax)

a. Embryo Experiment

1) Collection of Test Organisms. Fertilized E. mordax eggs were obtained from an adult brood stock maintained at the Southwest Fishery Center (NMFS) located in La Jolla, California. Brood stock adults were artificially induced to spawn following the methods of Leong (1971). Eggs were transported to MBC's bioassay facility within 5 to 8 hours after fertilization, where they were sorted to remove dead or non-viable eggs and placed in experimental chambers for the experiment.

2) Experimental System and Protocol. The embryo exposure experiment was conducted in one liter screw-cap glass jars. Six replicate jars were employed for the control and each of the three WSF concentrations. Nine hundred milliliters of the appropriate WSF test solution or control seawater, in addition to radioactive naphthalene tracer (Section B.2.a)1), Radioactive Tracer) were placed in each replicate test jar. Seventy developing embryos in the early blastula stage were placed in each replicate test jar. Plastic caps with paper liners were placed on the test jars and sealed with teflon tape to prevent the

loss of volatile hydrocarbons. Water quality parameters including dissolved oxygen, pH, salinity, and temperature of the source water were measured prior to initiating the experiments. Replicate test jars containing embryos were maintained for 48 hrs under constant temperature (16.0±0.5°C) and light (12 L:12D).

The embryo experiment was terminated 24 hrs after hatching began. Newly-hatched larvae from each replicate were anesthetized with tricaine methanesulfonate (MS 222) and pipeted from the culture jars. Larvae were then counted and 20 randomly selected individuals measured to the nearest 0.01 mm using an occular micrometer. All abnormally developed larvae were enumerated and the type of abnormality was noted. The total number of dead embryos and larvae present in each replicate was also recorded. Radioactive naphthalene uptake was determined for 10 to 15 larvae from each replicate (Section B.2.a) l), Radioactive Tracer). Any remaining live larvae were preserved in 4% formalin-seawater for histological examination (Section B.2.b)1), Histopathology).

b. Larval Experiments

1) Collection of Test Organisms. Fertilized E. mordax eggs were obtained from the Southwest Fishery Center (NMFS). Fertilized eggs were returned to MBC and held in aerated aquaria for approximately 36 hours at 16°C prior to use in larval experiments.

2) Experimental System. The experimental system employed in the E. mordax larval experiments was identical to that used for P. californicus (Section 3. b.2)).

3) Experimental Protocol. The larval experiment (Figure II-6) was initiated by placing 75 embryos that were close to hatching in each test chamber. Embryos, rather than newly-hatched larvae were transferred to the test chambers to minimize damage and stress due to handling. To monitor developmental progress and minimize handling stress, a second group of larvae was held under static conditions. Additional embryos (approximately 40 to 50) were held under static conditions in 0.5 liter petri dishes at 16.0±0.5°C until hatching. Larvae hatched from these eggs were anesthetized and measured to provide baseline length data on newly hatched larvae for evaluation of growth during the experiments.

Three replicate chambers were randomly sacrificed from the medium and low NSF concentrations and the seawater control on day 7. Day 7 was selected because it was just prior to the period when non-feeding larvae would die of starvation. All high concentration replicates were sacrificed on day 4 and the remaining medium concentration replicates were sacrificed on day 10 due to high mortality. On day 14 all remaining low concentration and control replicates were sacrificed. Upon termination of individual replicates, larval mortality, growth, radiotracer uptake and development were assessed and samples were obtained for histopathology.

4) Larval Feeding. Larval E. mordax required two different types of food depending on their development stage. Prior to complete yolk-sac absorption (day 2), the unarmored dinoflagellate Gymnodinium splendens was added to each experimental chamber. Sufficient G. splendens was added to achieve a concentration of approximately 200 cells/ml as suggested by Lasker et al. (1970}. The desired amount of G. splendens was placed in larval feeders,

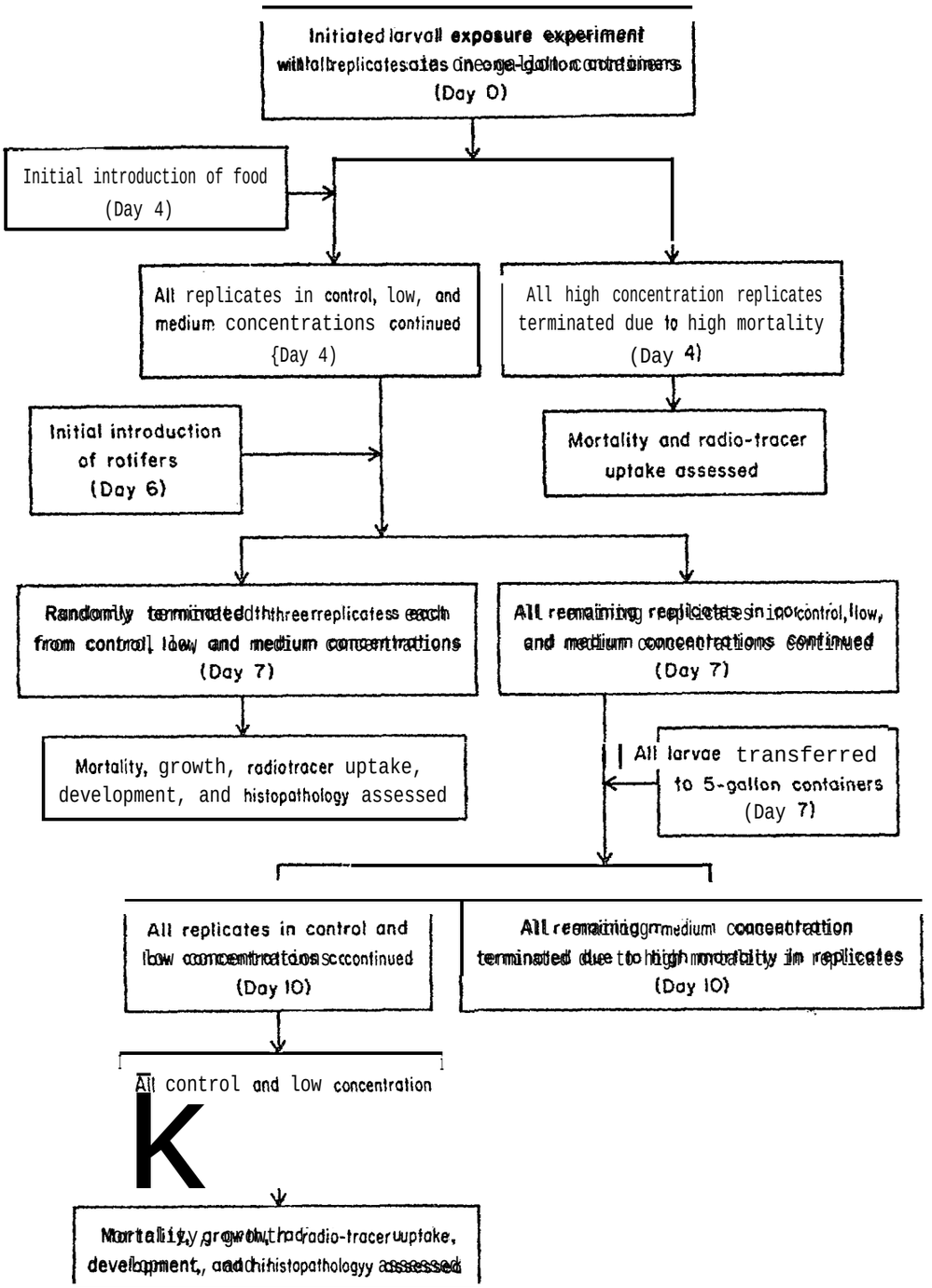


Figure II-6. Flow diagram depicting experimental protocol employed in larval Engraulis mordax exposure experiments.

which delivered food through Tygon tubing at a rate of 5 to 10 ml/min for approximately one hour. When food was delivered, it was necessary to discontinue the flow of test solution to the experimental chambers to reduce disturbance of the larvae. No G. splendens was added to the chambers following the initial introduction of food. Within two hours of food introduction, the flagellates migrated towards the surface forming a dense aggregation of cells at the water/air interface. This aggregation remained for 3 to 4 days during which time the E. mordax larvae concentrated near it and fed continuously.

On exposure day 5, larval E. mordax were fed the rotifer Brachionus plicatilis as recommended by Theilacker and McMaster (1971). The procedures used were identical to those employed in the larval halibut experiment (Section 3.b.3)).

5) Daily Monitoring and Observations. Daily monitoring and observation procedures were identical to those employed in the larval P. californicus experiment (Section 3.b.4)).

c. Adult Experiment

1) Collection and Holding of Test Organisms. Adult northern anchovy were obtained from San Francisco Bay by the Meatball Bait Distributing Company. Fish were obtained by seine in early August 1982 and held in open water holding pens. A commercial hatchery truck, fitted with a 400 gallon water tank, transported the fish to the University of California Santa Cruz marine laboratory in two separate lots (approximately 2,500 fish/lot). The truck was equipped with a splash system to maintain dissolved oxygen saturation and create a 2 to 3 mph current for fish orientation during transport to minimize loss. Total transport time from San Francisco to the Santa Cruz marine laboratory was 3 hrs. At the laboratory, fish were distributes among 12 circular fiberglass tanks (360 gallon) in approximately equal numbers for acclimation. All fish were maintained in these tanks throughout both the acclimation and experimental periods.

Each tank was treated with the antibiotic oxytetracycline (Terramycin™) at 25.0 mg/l to reduce the possibility of infectious bacterial outbreaks. Antibiotic treatments were continued for seven consecutive days (at 25.0 mg/l) during the acclimation period as a preventative measure. Other prophylactic measures included a single malachite green treatment at 0.5 mg/l for one hour to inhibit fungal growth and a formalin treatment at 80.0 mg/1 for one hour as a precaution against ectoparasites (Van Duijn 1973).

The acclimation period was extended from 2 to 3 weeks to permit recovery from stress of capture, handling, transport, and medication. The three week holding period was also necessary to allow the mortality loss to stop.

Several days prior to the start of the experiment, photographs were taken of the fish in each tank to determine the total number. Based on the photo counts, fish were then randomly redistributed to obtain an equal number of fish per tank. At initiation of the experiment, there were 255 individuals in each tank.

2) Experimental System. The experimental system used in the adult anchovy experiment was identical to that employed in the adult P. californicus experiment (Section 3.c.2)).

3) Experimental Protocol. The E. mordax experiment began in late August 1982. Before the experiment.. 50 fish were sacrificed and sexed. These fish were weighed to the nearest tenth of a gram and measured for both standard and total length to the nearest millimeter. This same group of 50 fish was then analyzed for background hydrocarbon tissue burdens. The 50 fish were divided into 10 replicate groups of 5 fish each. Each replicate group was separated into 1) head/viscera and 2) body muscle subgroups. The head/viscera of five fish were dissected, weighed, and combined to form half of one replicate. The remaining muscle tissue of the five fish was also weighed and combined to make up the other half of the replicate. All tissue samples were wrapped in contaminant-free aluminum foil (hexane-rinsed and oven baked), labeled, and frozen at -20°C. Samples were subsequently packed with a commercial coolant (Blue Ice™) and air-freighted to Science Applications, Inc. (SAI) in La Jolla, California for chemical analyses of petroleum hydrocarbons.

To determine the time course of changes in 1) growth, 2) hydrocarbon uptake, and 3) the onset of gross/histopathological abnormalities, a subsample of live E. mordax was removed every 30 days during the experiment. The subsampling procedure involved random removal of 12 fish from each replicate tank of a given concentration and the control. These fish were then composite to form samples of 36 animals per concentration. All subsampled fish were weighed to the nearest 0.1 gram, measured to the nearest millimeter for standard and total length, and examined for abnormalities. Twelve of the 36 anchovies were fixed whole in 10% buffered formalin and shipped to Dr. S. R. Wellings at the University of California, Davis, for histopathological processing and evaluation. The remaining 24 animals were prepared for tissue burden analysis and shipped to SAI as described previously.

Fish that died during the experiment were removed, weighed (to nearest 0.1 gram), measured and examined for abnormalities. Each fish was then incised along the ventral margin of the body cavity for preservative penetration to the internal organs and placed in 10% buffered formalin for subsequent autopsy.

4) Daily Observations and Monitoring. To ensure that test solution concentrations remained at targeted levels, the volatile hydrocarbon fraction was monitored daily in each tank (Section B.1.b.2)c), Aqueous Test Solution Samples). Flowmeter adjustments were then made as necessary to maintain the desired test solution concentrations. Weekly water samples from each test concentration were shipped to SAI for analysis of the extractable hydrocarbon fraction (Section B.1.b., Analysis of Parent Crude Oil and Test Solutions).

Four water chemistry parameters were measured daily within each tank: pH, dissolved oxygen, temperature, and salinity. During the experiment, daily observations were made on each fish in each tank, and any behavioral changes or abnormalities recorded including changes in coloration, schooling, feeding, and swimming behavior.

5) Feeding. Fish in each tank were fed approximately 2% of their body weight daily. Food consisted mainly of ground trout chow (Ralston-Purina Company) at the manufacturer's recommended feeding level for maximum growth. The total daily ration was administered four to five times during the day. Trout chow was periodically supplemented with flake food (TetraMarin) and with live or frozen brine shrimp. Antibiotic medication (oxytetracycline) was also occasionally administered with the brine shrimp at a rate of 1.8 mg/gm of food (Van Duijn 1973). Tanks were scrubbed and cleaned daily to remove feces, excess food, debris, and algae.

5. CALIFORNIA MUSSEL (Mytilus californianus)

a. Embryo Experiment

1) Collection of Test Organisms. Gametes for the M. californianus exposure experiment were obtained from mussels collected at Crescent Bay, Corona del "Mar, California. In the laboratory, shells were cleaned of all foreign material and the mussels maintained without seawater at 9°C (45°F) for 48 hrs.

Spawning was initiated by transferring mussels from aquaria at 9°C to seawater at 20°C. Following spawning, fertilized eggs were removed from the aquaria and passed through multiple screens (mesh size 125, 100, 75, 50, and 20 microns) to remove excess sperm and foreign material. Eggs retained on the 20 micron screen were washed with fresh seawater and placed in a four liter glass holding aquarium.

Six replicate 900 ml static culture jars were used in the embryo experiment per test concentration. Test jars were the same as those employed in the P. californicus and E. mordax embryo studies (Sections B.3. and 4 al), Collection of Test Organisms).

2) Experimental System and Protocol. Embryos placed in the holding aquarium were lightly aerated to ensure even distribution of embryos throughout the container. Their density within the container was determined by calculating the mean number of embryos found in 20 one m? aliquots ($\bar{X}$=177 .7±13.4 embryos/ml).

Approximately one hour after fertilization, embryos were randomly deposited in the test jars (approximately 332±17 per jar). The experiment was conducted under static conditions. Following an initial dosing of embryos with the appropriate test solution (i.e. 5, 50, or 500 ppb WSF) and innoculation with 0.3 ml of radiolabeled tracer, the jars were capped and sealed with tape. This procedure was used to prevent subsequent loss of the volatile test solution components. The experiment was conducted under indirect light conditions (12 hr L:12hr D cycle) at a temperature of 16.0+0.5°C.

Experimental exposure was allowed to continue until the embryos had developed into the veliger stage (approximately 96 hrs). Upon termination of the experiment, 10.0 ml of neutral red vital stain (Crippen and Perrier 1974) was added to each jar to assist in mortality determination. The larvae were kept in the stain for one hour after which the content of each jar was filtered through a 20 micron screen. Retained larvae were washed into a counting dish containing a 5% solution of magnesium chloride anesthetic in seawater. The vital stain not only stained live larvae, but also stained dead larvae various shades of light red to yellow. For the purpose of this investigation, only larvae that stained a dark red were considered alive. Not all larvae had transformed into veligers when the investigation was terminated. Those trochophores encountered were also counted.

Data gathered from the embryo investigation included the number of 1) live and dead veligers, 2) live and dead trochophore larvae, and 3) malformed larvae. Veligers for use in the radioactive tracer uptake analysis (20 veligers from each replicate) were separated and prepared following the procedures outlined in Section B.2.a. (Radiotracer Technique). Remaining live larvae were preserved in 4% buffered formalin-seawater for histological examination.

3) Water quality measurements (i.e. temperature, dissolved oxygen, pH, and salinity) of the test solutions were determined on the source waters prior to placing the WSF test solution in the culture jars.

b. Larval Experiment

1) Collection of Test Organisms. California mussel gametes were obtained following the procedures outlined in Section B.5.a.1), Collection of Test Organisms.

Following spawning, fertilized eggs were removed from the aquaria by passing the gametes through multiple screens (mesh sizes 125, 100, 75, 50, and 20 microns) to remove excess sperm and foreign material. Eggs retained on the 20 micron screen were washed with fresh seawater then distributed to holding aquaria.

2} Experimental System. Nine replicate aquaria per test concentration were used in the M. californianus study. The aquarium jars were the same as those employed in the P. californicus and E. mordax investigations (Section B.3.b.2) California Halibut Experimental System). The mouths of the culture aquaria were covered with 39 micron mesh nitex screen. The screen prevented loss of larvae but allowed for circulation of seawater and test solution through the system.

3) Experimental Protocol. Fertilized eggs were placed in holding aquaria and maintained under static conditions at 16+0.5°C until metamorphosis into veligers (approximately 72 hrs). Veligers were removed from holding aquaria by filtering the aquarium contents through a 20 micron nitex screen. The larvae were then transferred to a holding tank and allowed to acclimate for 2 hrs. Following acclimation, those veligers not swimming in the water column were siphoned from the tank bottom and discarded.

Veliger holding tanks were lightly aerated to evenly distribute larvae throughout the water column. Their density within the holding tank was determined by calculating the mean number of individuals found in twenty 1.0 ml aliquots. The animals were transferred to experimental aquaria by siphoning approximately 65,200 veligers out of the holding tank and depositing the veligers in randomly selected aquaria. Length and height measurements to the nearest. 0.1 mm were made on these organisms to provide baseline data for future growth determinations.

Veliger response to WSF test concentrations was monitored by removing replicates at discrete time intervals (Figure II-7). The experiments were initiated with nine aquaria per test concentration. Three replicates from each concentration were scheduled for termination at days 7, 14 and 31 of the experiment. On day 7 three replicates were removed as scheduled. However, a sharp rise in mortality on day 11 in the high concentration necessitated termination of all remaining replicates in this concentration. To provide comparative data at this time five 1 ml aliquots were removed from each remaining aquarium (control, low and medium concentrations). The number of larvae removed from each replicate is provided in Table II-4.

Larval replicates sacrificed from all concentrations were examined for mortality, radiotracer uptake, growth and histopathology. Three replicates from each of the remaining concentrations were terminated on day 14. Between exposure days 23 and 24, a major decline in veliger densities was noted. On

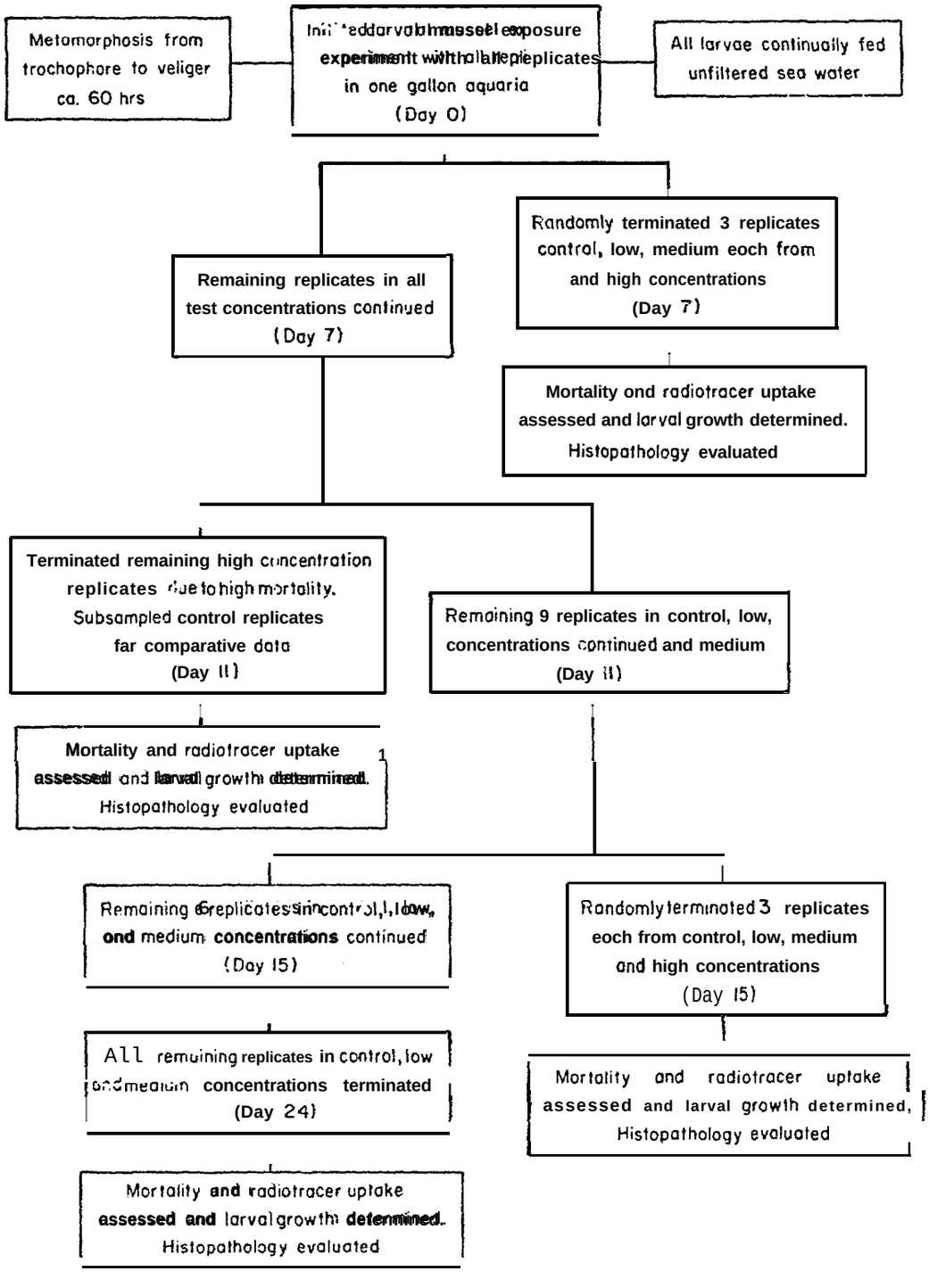


Figure II-7. Flow diagram illustrating experimental protocol employed in larval Mytilus californianus exposure emperiments.

exposure day 24, the remaining three replicates in the control, low, and medium test concentrations were terminated.

Table it-4. Total number of larvae removed from the six control replicates on larval *exposure* day 11.

Replicate Number	Number of Larvae Removed
D-2	53
0-3	28
D-4	31
D-6	32
D-8	19
D-9	32

Termination of an exposure aquarium involved the following steps:

a) The aquarium jar top was sealed with a stopper and the jar slowly turned over until the screened mouth was upright.

b) The jar was transferred out of the holding tank and the nitex screen removed. The volume of the culture jar was brought to three liters and the contents lightly aerated to ensure even distribution of the veligers.

c) Density of the veligers within each culture jar was estimated by removing three subsamples. The volume of the subsample was dependent on the density of the veliger population remaining in the aquarium. Initially, a 1 ml subsample was extracted from the aquarium. If no animals appeared in any one of the three subsamples, the volume was increased first to 100 ml and then to 500 ml. If veligers were not present in a 500 ml subsample, the entire contents of the aquarium jar was filtered through a 20 micron mesh screen and a total count of the live veligers made. Subsample volumes for each density estimated are presented in Table II-5.

d) Following veliger density estimates, the entire contents of the aquarium were filtered through a 20 micron mesh screen. The material retained on the screen was washed into a petri dish and live veligers were separated from debris.

e) Veligers for use in the radioactive tracer uptake analysis were removed and the remaining veligers preserved in 4% buffered formalin-seawater. The number of veligers/replicate used for the radiotracer uptake analysis was dependent on the number of live veligers available in the sample. Numbers ranged from 10 to 50 veligers/replicate (Table II-6).

Table II-5. Subsample volumes used for veliger density estimates.

Test Concentration	Termination Data Subsample Volume
Exposure Day 7	
Control	1 ml
Low	1 ml
Medium	1 ml
High	100 ml
Exposure Day 11	
Control	1 ml
High	*
Exposure Day 15	
Control	100 ml
Low	100 ml
Medium	100 ml
Exposure Day 24	
Control	500 ml
Low	500 ml
Medium	*

* Veliger density determined by counting total remaining live larvae.

f) Preserved larvae were examined for histological abnormalities (see Section B.2.b., Histopathology Methods).

g) The length and height of 20 randomly selected veligers were measured to the nearest 0.1 mm for growth evaluation using a compound microscope fitted with an ocular micrometer.

4) Larval Feeding. Veligers were allowed to feed on algae, bacteria

and other material contained in the unfiltered seawater. Microscopic examination of seawater supplied for the aquaria revealed that an unidentified chlorophyte alga approximately 2 to 3 micron in diameter was present at a density of 10 to 50 cells/microliter. Further examination of veliger gut contents confirmed that larvae were feeding on the chlorophyte alga and other material. Density of the unidentified chlorophyte within the culture jars was monitored daily. These determinations were made at the same time veliger density estimates were made. It was not known if the unfiltered seawater provided all the nutritional requirements of the veligers.

Table II-6. Number of veligers used in radioactive tracer uptake analysis.

Concentration	Exposure Period	Veligers/ Replicate
	Exposure Day 7	
Control		50
Low		50
Medium		50
High		50
	Exposure Day 11	
Control		10
High		20
	Exposure Day 15	
Control		50
Low		50
Medium		50
	Exposure Day 24	
Control		50
Low		50
Medium		10*

* Only two of the three replicates with live veligers.

5) Daily Observations. The small size of M. californianus veligers (length <200 microns) precluded daily observations. However, gross estimates of veliger densities were made daily. One milliliter aliquots from each replicate were counted on microscopic slides, and then washed back onto the source aquaria. Water quality measurements were made as in other larval experiments (Sections B.3. and 4.b.5, Daily Observations and Monitoring).

c. Adult Experiments

1) Collection of Test Organisms. Adult Mytilus californianus were collected by hand at Bonny Doon Beach, Santa Cruz, California. In the laboratory all adhering organisms and debris were removed from the exterior surface of the mussel shells. The animals were then placed in 360 gallon holding aquaria with flowing seawater.

2) Experimental System. Mussel experiments were conducted concurrently with those for adult California halibut. The experimental system was described previously [Section B.3.c.2)]. In order to use the entire water column within the experimental tanks and to minimize interference with demersal halibut, the mussels were suspended in plastic racks {which had been previously aged in seawater) within each tank.

3) Experimental Protocol. Upon arrival at the Santa Cruz facility, mussels were placed in 360 gallon tanks with continually flowing seawater and allowed to acclimate and depurate for three weeks. Following acclimation four hundred animals were tagged with 5 mm ($^1/4$") diameter Petersen numbered discs bonded to their shell with Z-Spar Splash Zone Epoxy (A-788). The animals were returned to holding tanks for two weeks to allow epoxy to cure.

Background hydrocarbon levels from 50 animals were measured in target organ tissues including the gill, gonad, digestive gland and muscle (retractor and adductors) prior to beginning the experiments. Tissues were separately analyzed by GC and GC/MS [Section B.2.2) Tissue Extracts] to identify and determine petrogenic hydrocarbon levels.

Prior to initiating experiments, animal shell length, height, and width were measured and recorded for future comparison. Animals were then randomly assigned to experimental tanks. Three toxicant levels plus control were tested. Thirty animals were placed in each tank, three replicate tanks per concentration {i.e. 90 animals/concentration), a total of 360 mussels tested.

Animals were exposed to three target concentrations of hydrocarbons (e.g. 5, 50 and 500 ppb) as in all experiments. Exposure duration was 120 days.

Following the 120 day exposure period, those animals remaining were sacrificed for histological examination and chemical analyses. Dissected tissues from 50 animals were subjected to histological examination (Section B.2.b, Histopathology) and the remainder were chemically analyzed for hydrocarbon tissue burdens [(Section B.2.a.2), Tissue Extracts].

4) Daily Observations and Monitoring. Dissolved oxygen, pH, temperature, salinity and test solution concentrations were monitored daily. The measurements were performed on one replicate tank from each toxicant level tested plus control. Different replicate tanks were measured on successive days to ensure all were supporting similar exposure regimes.

During the experimental period, several parameters were monitored. On a daily basis, valve closing response to handling stimuli, mortality, retraction of foot, byssus thread production, and general health were noted. On a weekly 'oasis, byssus threads from 30 individuals (one per replicate) in each concentration were cut and counted. Animals which had expired were wrapped in hexane-rinsed foil and frozen for histological evaluation and chemical analyses.

5) Feeding. Adult mussels were fed daily. At feeding time, unfiltered seawater was substituted for the normally filtered seawater. This procedure ensured that the animals received food greater than 45 micron which was normally filtered out by the marine lab system. The animals were allowed to feed for two hours before the system was switched back to unfiltered seawater.

6. STATISTICAL ANALYSES

Various statistical procedures were applied to data generated by each experiment. The tests compared results from experimental aquaria with one another as well as with controls. Replication by design allowed for rigorous statistical comparisons.

The Kruskal-Wallis (Sokal and Rohlf 1969) one-way analysis of variance (K-W ANOVA) was used to test for mean differences between the control and various exposure levels. When a significant difference was found, a non-parametric version of the Student-Newman-Keuls multiple range test (SNK test) was used to determine which means differed. In addition, each experimental treatment group was compared with the control group utilizing Dunnett's test (Steel and Torrie 1960). A significance level of 0.05 was used in all statistical tests.

Use of the non-parametric K-h' ANOVA and SNK tests did not require any assumption of normality in the sampling error distribution (Conover and Iman 1981), and resulted in little loss of power. Although a slight amount of

power can be lost when sampling error is normally distributed, the power of the K-W ANOVA test has up to 95.5% of the power of the parametric ANOVA (Siegel 1956). With regard to multiple comparison tests, Conover and Iman (1981) state that "The power with normal populations is about the same whether the analysis is done on the data or on the ranks." When the errors are not normal , the non-parametric tests are more powerful and robust. The possible loss of a small percentage of power in these studies was balanced by the greater risk of non-normal errors.

Chi-square tests were utilized in some embryo and larval experiments to determine if there were significant differences in hatching success and the incidence of developmental abnormalities.

More detail on these tests can be found in Zar (1974), Sokal and Rohlf (1969), and Steel and Torrie (1960).

C. RESULTS

1. TEST OILS

a. Crude Oil Characterization

Santa Barbara crude oil was the parental toxicant used in all chronic bioassay experiments. Generically, this material was a sour crude (i.e. no chemical or physical treatment prior to the time it was obtained) produced from Monterey Zone formations located off the coasts of Santa Barbara and Ventura Counties in the Southern California Outer Continental Shelf (OCS). The specific crude used in these studies was obtained from lot 9 of the oil produced on Platform Holly; well number 3120. The oil was collected into 55 gallon plastic drums directly from the offshore pipeline prior to any form of preliminary treatment. The drums were subsequently sealed and shipped to the site of the bioassay studies.

Crude oils are a naturally occurring complex mixture of both inorganic and organic constituents. Because of the inherent complexity of the primary toxicant, initial chemical studies were focused on characterization of the Santa Barbara crude oil. The crude oil characterizations were performed to define the specific chemical compounds to which the organisms were exposed, as well as to determine the composition of Santa Barbara crude relative to other crude oils.

Table 11-7 presents data on gross characterization parameters for Santa Barbara crude and four other crudes which are produced and/or transported in the marine environment. The gross characterization parameters suggest that Santa Barbara crude has a composition which is typical of a mixed-base crude, and representative of crudes produced in the marine environment. Santa Barbara crude is characterized by a relatively high specific gravity, high viscosity and substantial asphalt content. The most distinguishing characteristic of the Santa Barbara crude is its high sulfur content, which is typical of "sour crudest'.

In addition to the whole crude physical property characterizations, detailed characterizations of the organic constituents were performed on the Santa Barbara crude. As described previously, GC and GC/MS techniques were used in conjunction with sample fractionation procedures for elucidation of

Table H-7. Gross characterizations of selected whole crude oils.

Crude Oil	API Gravity	Specific Gravity (g/ml)	Viscosity (100°F) Kinematic CST	Viscosity (100°F) Saybolt SUS	Pour Point["F]	% Asphalt	Ni (ppm)	V (ppm)	S (%)	N (%)
Santa Barbara, CA*	21.1	0.927	97	450	-25	42	30	163	3.8	0.5
Wilmington, CA**	19.4	0.938	100	470	5	24	100	81	1.8	0.8
Murban, Abu Dhabi**	40.5	0.829	3	36	-20	7	3	10	1.0	0.1
Cook Inlet, Alaska**	35.4	0.848	17	85	-15	12	1	0.5	0.1	0.1
Prudhoe Bay, Alaska**	27.0	0.893	19	84	-10	23	14	28	1.0	0.3

● Data obtained from producer.
● * Data adapted from Payne et al . 1981.

compound specific composition. The analytical protocol resulted in characterization of four distinct crude oil fractions, including: volatile, aliphatic, aromatic and polar/heterocyclic components.

The results of these detailed characterizations are presented in Tables 11-8 through 11-11. A qualitative summary of the major volatile crude components is given in Table II-8, and quantitative data are presented in Tables II-9, II-10 and II-11 for the components identified in the aliphatic, aromatic and polar/heterocyclic fractions, respectively. Figure II-8 presents the GC/MS reconstructed ion chromatogram for the volatile crude constituents, which corresponds to the tabular summary presented in Table II-8. The capillary column gas chromatograms corresponding to the aliphatic, aromatic and polar/heterocyclic fraction tabular summaries are presented in Figures II-9a-c, respectively.

As evidenced by the chromatograms in Figures II-8 and II-9, Santa Barbara crude oil is a very complex mixture of organic constituents. This crude is characterized by a volatile fraction composed of aliphatic, cyclic and aromatic components. As indicated in Table II-8, the volatile aliphatics include C5 through C_{11} normal alkanes, and a series of alkyl-substituted homologs. Similarly, a full spectrum of cyclic hydrocarbons were identified, including C_5 through C_7 parent hydrocarbons and a wide variety of alkyl-substituted homologs. The volatile aromatic species are dominated by benzene and its alkyl-substituted homologs, as well as several thiophenes. These

Table II-8. Qualitative GC/MS analysis of volatile components in Santa Barbara crude oil (see Figure II-8).

Aliphatic Hydrocarbons	Cyclic hydrocarbons	Aromatic Hydrocarbons
nC-5 [1]*	Cyclopentane (2)	benzene (7A)
Dimethylpentane (4.6)	Methyl cyclopentane (5)	Toluene (9A)
Trimethylpentane (15]	Ethylcyclopentane (121	Dimethylbenzene (32,35)
nC-6 {3)	Trimethylcyclopentane (13,14)	Ethylbenzene (31)
Methylhexane [2,8)	Ethyl -methyl cyclopentane (20,21,24)	Methyl-etnylbenzene {43,46}
Dimethylhexane (16!		Methylethylbenzene [39)
nC-7 (10)	Cyclohexane (7)	Trimethylbenzene (48,50)
Methyl heptane (17)	Methyl cyclohexane (11)	Ethyl dimethylbenzene (52)
Oimethylheptane [26,27,30}	Dimethylcyclohexane (18,19,22]	
Methyl -ethyl heptane (42,51)	Ethyl cyclohexane (28)	
nC-8 (23)	Trimethylcyclohexane (.29,34)	
Methyloctane (33)	Methyl -ethyl lcyclohexane (38)	
Dimethyloctane (41,44)	Propy lcyclohexane (40]	
nC-9 (361	Methyl -propylcyclohexane (47}	
Methylnonane (45)		
nC-10 (49)	Cycloheptane (9)	
nC-11 (53)		

● Number(s) in parenthesis indicates peak number on Figure I I-8.

Table II-9. GC/MS identification of selected components in the aliphatic fraction of parental Santa Barbara crude oil (see Figure n-9a).

Peak Number	Compound	Cone. (mg/g)
1	nC-8	2.2
2	nC-9	2.8
3	nC-10	2.4
4	nC-11	2.4
5	methyl decane	0.7
6	nC-12	2.4
7	methyl -ethyl heptane	1.0
8	methyl -ethyl heptane	0.6
9	dimethyloctane	1.3
10	methyl nonane	0.3
11	nC-13	2.1
12	methyl undecane	1.3
13	nC-14	1.9
14	dimethyl undecane	1.4
15	nC-15	2.2
16	nC-16	2.0
17	nC-17	1.5
18	pristane	1.1
19	nC-18	1.7
20	phyta ne	1.6
21	tetramethyl heptadecane	0.3
22	nC-19	1.4
23	nC-20	1.8
24	nC-21	1.4
25	nC-22	1.3
26	nC-23	1.2
27	nC-24	1.1
28	nC-25	1.1
29	nC-26	1.0
30	nC-27	1.1
31	nC-28	0.6
32	nC-29	0.6
33	nC-30	0.5
34	nC-31	0.4
35	nC-32	0.4
	Total n-Alkanes	39. t
	Total Resol ved Hydrocarbons	93.7
	Total Unresolved Hydrocarbons	76.3
	Total Hydrocarbons	170.0

Table 11-10 . GC/MS identification of selected components in the aromatic fraction of parental Santa Barbara crude oil (see Figure 11-9 b).

Peak Number	Compound	Cone. (mg/g)
1	tol uene	2.6
2	etnyl benzene	0.4
3	p-xylene	0.8
4	o ,m- xyl ene	0.4
5	cumene	0.1
6	n-propyl benzene	0.1
7,9	ethylmethylbenzene	0.7
8	mesi tyl ene	0.2
10	trimethylbenzene	0.5
11	p-cymene	0.3
12,15,18	methyl propyl benzene	0.4
13	n-butylbenzene	0.1
14,16,17	ethyl dimethyl benzene	0.5
18,19,21	tetramethyl benzene	0.1
20	dime thylpropylbenzene	0.1
22	naphthalene	0.2
23	benzothiophene	0.1
24	di hydrodimethyl-1 H-indene	0.1
25	n-hexyl benzene	•
26	2-methyl naphthal ene	0.6
27	ethyl tetrahydronaphthal ene	
28	I-methyl naphthalene	0.4
29,30	trimethyl -tetrahydronaphthal ene	0.3
31,33	dimethyl benzo/B/thi ophene	0.1
	2,6-dimethyl naphthalene	0.4
34,%,37	dimethyl naphthalene	0.2
35	ethyl benzo/B/thiophene	
38,40 ,41,42, 43,44,45	trimethyl naphthalene	0.5
39	methoxydimethyl benzofuran	0.1
46	diethyl benzo/B/thiophene	*
47	methoxybi phenyl	
48	dibenzothiophene	0.1
49,50	methyl dibenzothiophene	*
51,52	methyl phenanthrene	0.1
53.54	dimethyl naphtha/2,3 -B/thiophene	0.1
55,56	dimethyl phenanthrene	0.1
	Total Resolved Hydrocarbons	25.0
	Total Unresolved Hydrocarbons	120.6
	Total Hydrocarbons	145,6

● concentration <0.1 mg/g

latter compounds contribute significantly to the "sour" aromatic aroma characteristic of fresh Santa Barbara crude oil.

Table II-11. GC/MS Identification of selected components in the polar/ heterocyclic fraction of parental Santa Barbara crude oil (see Figure II-9c).

Peak Number	Compound	Cone. (mg/g)
1	Dimethylphenol	*
2	Trimethylphenol	*
3	Heptadecanol	•
4	nC-11	*
5	C16 acid	*
6	Substituted aromatic acid	*
7	nC-25	*
8	nC-28	•
	Total Resolved Hydrocarbons	4.9
	Total Unresolved Hydrocarbons	0
	Total Hydrocarbons	4.9

● Concentration less than 0.1 mg/gm.

The aliphatic fraction of Santa Barbara crude is characterized by a regularly repeating series of normal alkanes and branched and cyclic hydrocarbons. This pattern is characteristic of a number of crude oils, including many of those produced in Alaska, the Middle East and the Gulf of Mexico. The distribution of n-alkanes is presented graphically in Figure 11-10, along with those of selected other crudes. Santa Barbara crude has an n-alkane distribution in which C_8-C_{15} n-alkanes predominate, and the intensity subsequently declines toward the heavier n-alkanes. The odd/even n-alkane ratio is near 1.0.

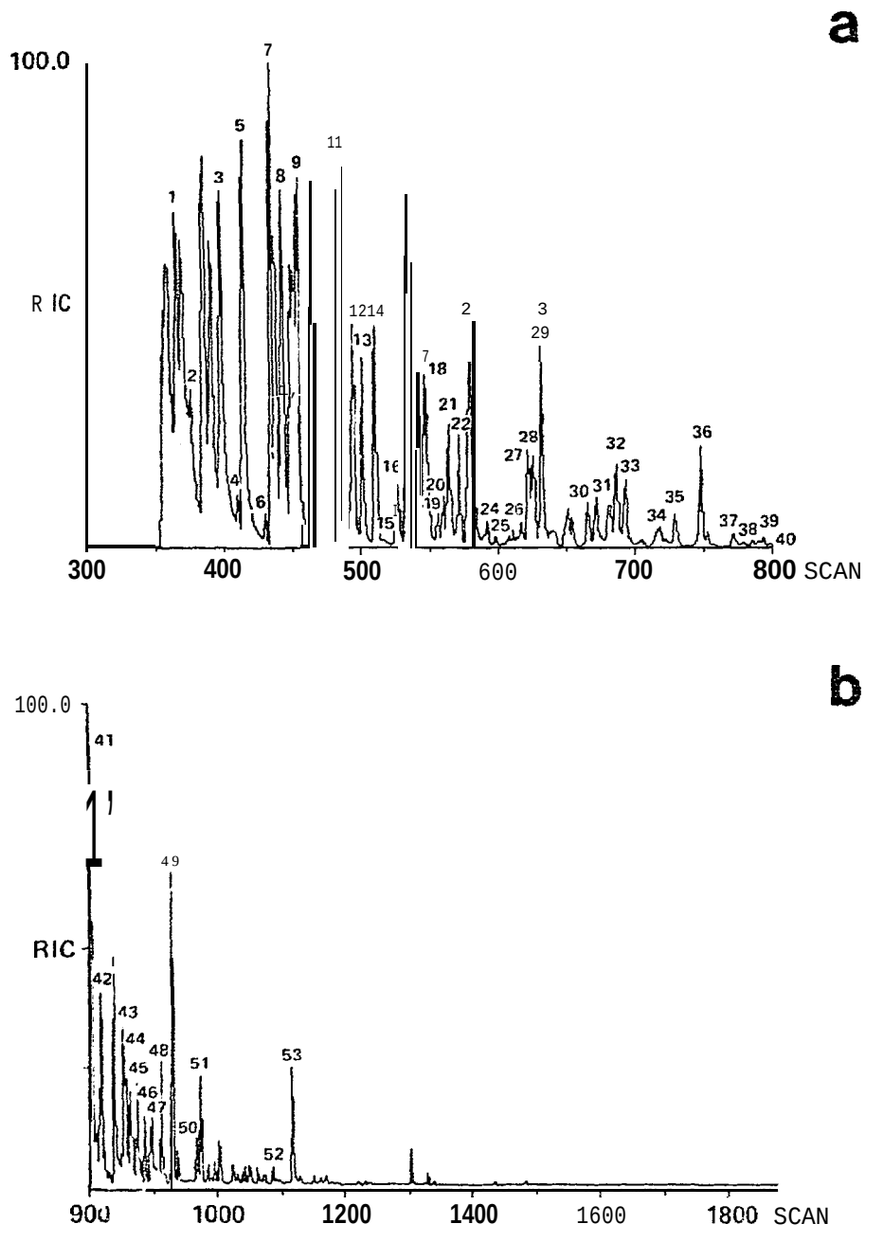


Figure II-8. Capillary GC/MS reconstructed ion chromatogram of volatile components in Santa Barbara crude oil: a) scan 300-800; b) scan 800-1900.

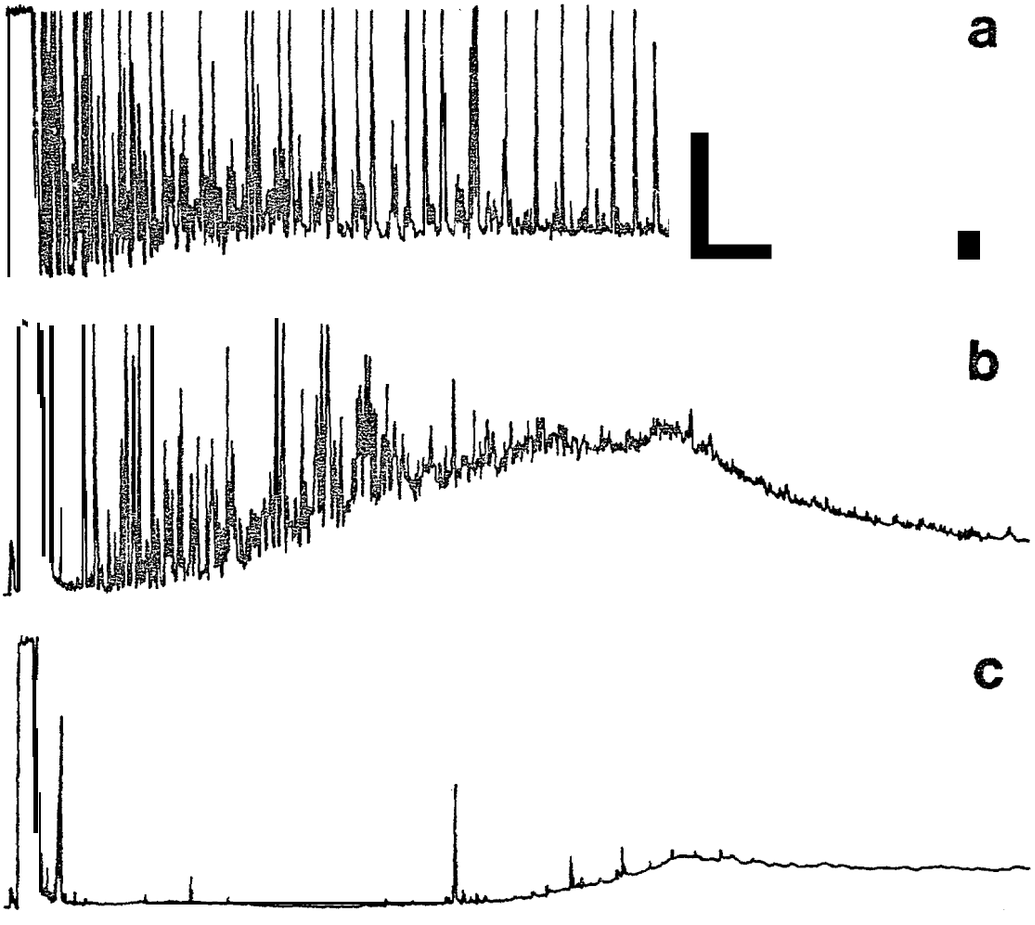


Figure II-9. *Flame* ionization defector *capillary gas chromatograms of* fractionated *Santa Barba crude* oil: a) aliphatic traction; b) aromatic fraction; c) polar/heterocyclic fraction.

Parent and alkyl-substituted benzenes, naphthalenes and phenanthrenes, as well as parent and substituted furans, pyrans and thiophenes were the predominant aromatic species identified in the Santa Barbara 'crude. Examination of the specific species identified (Table 11-10) indicates that a relatively extensive range of substituted homologs was observed. Of the predominate classes of aromatic compounds, the substituted benzenes and naphthalenes were found in both the greatest numbers and greatest concentration levels.

As is the case with most crude oils, relatively few chromatographable components were observed in the polar/heterocyclic fraction of the Santa Barbara crude (Figure II-9c). Compounds identified in this fraction include quinolines, pyridines, phenols and oxygenated heterocyclics. The polar/heterocyclic species represented only about 1.3% of the total non-volatile chromatographable crude oil components, a value characteristic of most crudes.

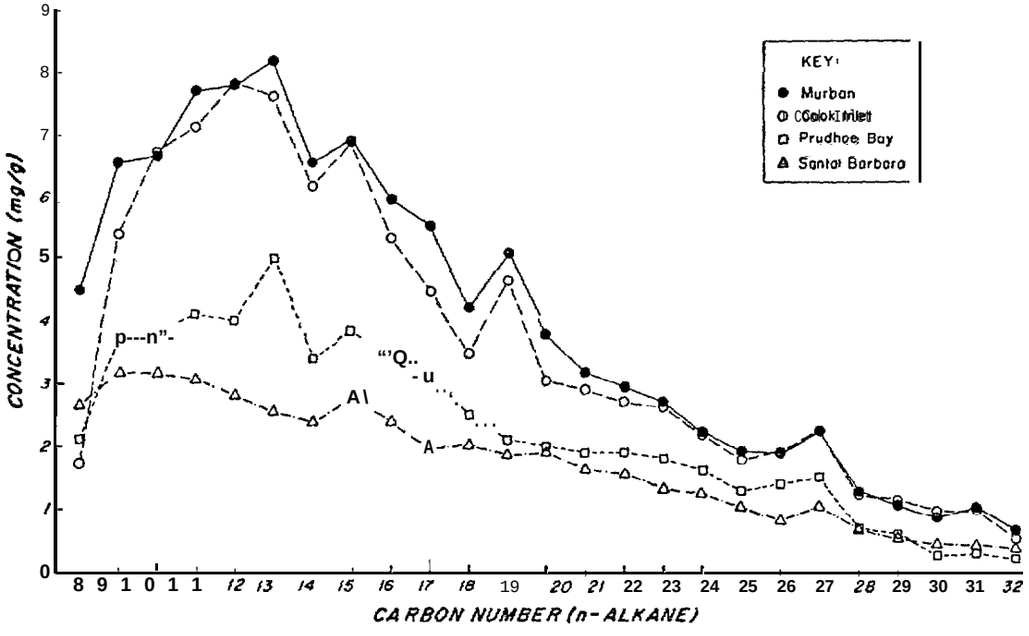


Figure II-10. Aliphatic (n-alkane) carbon distributions for Murban (Middle Eastern), Prudhoe Bay (Alaskan), Cook *Inlet* (Alaskan), and Santa Barbara (California) crude oils.

An evaluation of the consistency of the Santa Barbara crude oil was also a focal point of the chemical characterizations. Because of the complexity of the primary toxicant, there was concern that inherent variability might pose a problem in establishing a uniform WSF for the exposure of test organisms. To investigate this possibility, crude oil analyses were performed on samples collected from individual barrels obtained and used during different experiments. Results of these analyses indicated that the crude oil did in fact display a constant composition; however, some degree of variability was observed (i.e. in the total quantities of compounds). These changes could be attributed to weathering and analytical error associated with analysis of very complex matrices. Gas chromatograms of the aliphatic and aromatic fractions of crude oils used in the larval and adult experiments are presented in Figure 11-11. These chromatograms provide a visual indication of the compositional consistency of the Santa Barbara crude utilized, and further documentation is provided in the data appendix.

b. Test Solution Characterization

The initial step in preparation of the experimental exposure solutions involved generation of a concentrated stock solution of seawater soluble crude oil components. Results of the stock solution characterization are presented in Tables 11-12 through 11-14. The stock solution was fractionated prior to analysis. and Tables 11-12 through 11-14 present quantitative data for the components identified in the aliphatic, aromatic and polar/heterocyclic fractions, respectively. The capillary column gas chromatograms corresponding to these tabular summaries are presented in Figure II-12. As evidenced by these data, the crude solubilizer system produced a very complex water soluble fraction containing over a hundred individual organic compounds.

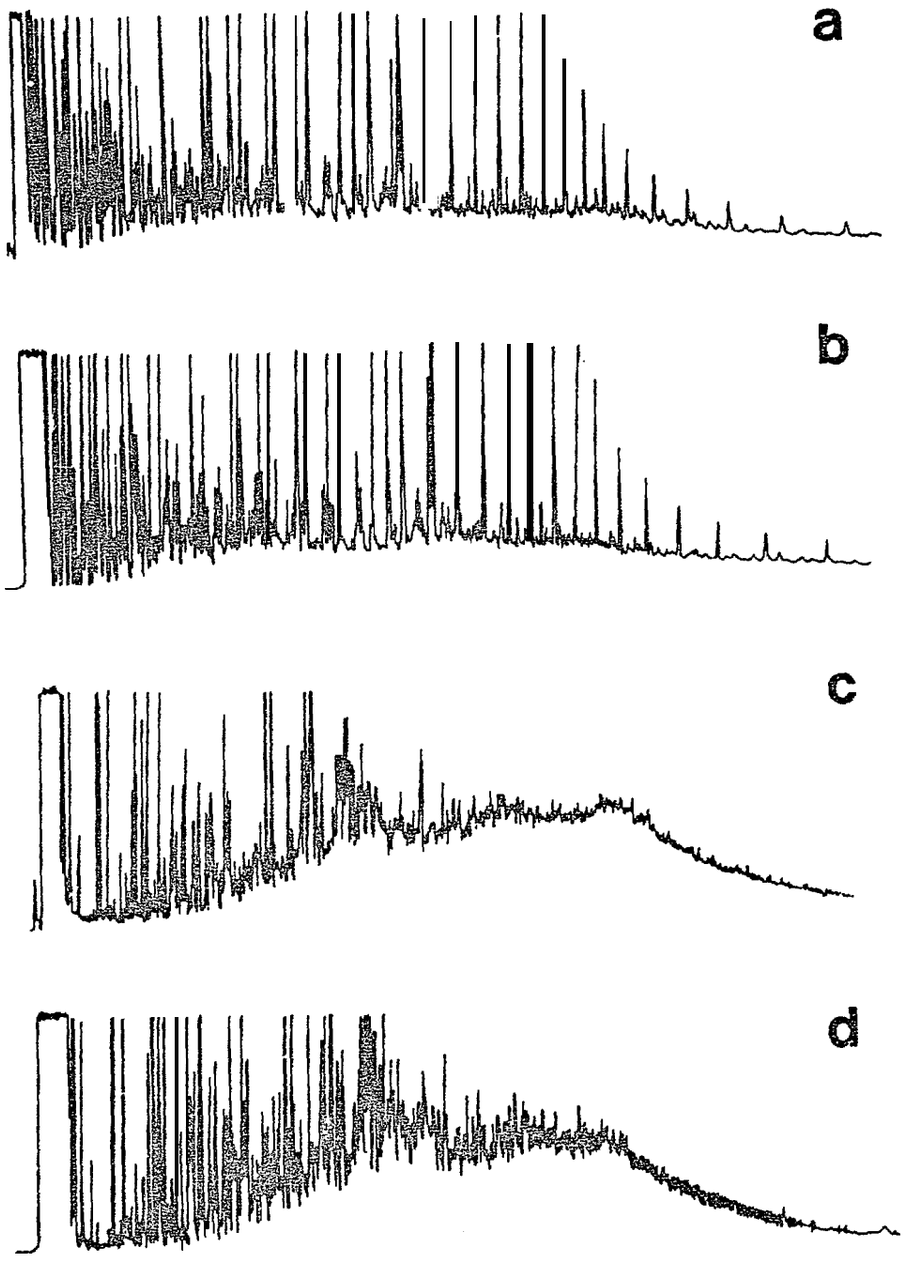


Figure II-11. Flame ionization detector capillary gas chromatograms of fractionated Santa Barbara crude oil: a) aliphatic fraction, larval study; b) aliphatic fraction, adult study; c) aromatic fraction, larval study; d) aromatic fraction, adult study.

Table II-12. GC/MS identifications of selected components in the aliphatic fraction of stock test solution (see Figure 11-12a).

Peak Number	Compound	Cone. (mg/g)
1	nC-8	0.2
2	nC-9	0.3
3	nC-10	0.4
4	nC-11	0.5
5	nC-12	0.4
6	Tetramethyloctane	0.2
7	nC-13	0.4
8	nC-14	0.4
9	nC-15	0.5
10	nC-16	0.5
11	nC-17	0.4
12	Pristine	0.2
13	nC-18	0.4
14	Phytane	0.4
15	nC-19	0.3
16	nC-20	0.3
17	nC-21	0.3
18	nC-22	0.3
19	nC-23	0.2
20	nC-24	0.2
21	nC-25	0.2
22	nC-26	0.2
	Total n-alkanes	6.4
	Total Resolved Hydrocarbons	14.9
	Total Unresolved Hydrocarbons	0
	Total Hydrocarbons	14.9

The aliphatic fraction of the stock test solution contained relatively few components and was dominated by a regularly repeating series of n-alkanes. The n-alkane species present were limited to those with chain lengths from 8 to 22 carbon atoms. The levels of these n-alkanes were low and very constant, ranging from 0.2 to 0.5 µg/l. Very few branched aliphatic and cyclic hydrocarbons were identified in the aqueous media, although a few low molecular weight species were observed. In general, the stock solution contained only low levels of relatively few aliphatic hydrocarbons due to the limited aqueous volubility of these compounds.

Alkyl-substituted benzenes, naphthalenes and thiophenes were the dominant components identified in the aromatic fraction of the stock test solution. A full spectrum of C_1 to C_4 substituted benzenes were identified, including all four C_2 substituted isomers. In all, 15 different substituted benzene isomers were identified, with individual

Table II-13. GC/MS identifications of selected components in the aromatic fraction of stock test solution (see Figure 11-12 b).

Peak Number	Compound	Conc. (mg/g)
1	Toluene	2.9
2	Ethyl benzene	10.8
3	p-Xylene	33.3
4	o,m-Xylene	9.7
5	Methyl ethyl benzene	4.3
6	n-Propylbenzene	4.6
7	Methyl -ethyl benzene	6.6
8	Mesitylene	3.6
9	Trimethyl benzene	11.1
10	Cymene	21.5
11	Methylethyl benzene	2.6
12	Methyl (methyl ethyl) benzene	3.2
13	Ethyl -dimethyl benzene	1.5
14	Ethenyl-ethylbenzene	0.5
15	Tetramethyl benzene	8.7
16	Naphthalene	12.1
17	Benzothiophene	2.1
18	2-Methyl naphthalene	10.5
19	Methyl naphthalene	0.5
20	1-Methylnaphthalene	8.2
21	Dimethylnaphthalene	2.0
22	Dimethylnaphthalene	0.3
23	Dimethyl naphthalene	1.0
24	Ethyl -benzo/B/thiophene	1.3
25	Substituted benzoic acid	0.3
	Total Resolved Hydrocarbons	793
	Total Unresolved Hydrocarbons	63
	Total Hydrocarbons	856

Table II-14. GC/MS identifications of selected components in the polar/ heterocyclic fraction of stock test solution (see Figure II-12c).

Peak Number	Compound	Cone. (mg/g)
1	Phenol	21.7
2	o-Methyl phenol	34.0
3	p-Methyl phenol	26.1
4	2,6-Dimethyl phenol	13.0
5	Ethylphenol	13.8
6	2,4-Dimethylphenol	36.7
7	Ethyl phenol	0.3
8	3,5-Dimethylphenol	1.4
9	3,5-Dimethylphenol	71.2
10	Dimethylphenol	1.5
11	Dimethylphenol	1.4
12	2,4, 6-Trimethylphenol	9.2
13	2,3,6 -Trimethylphenol	24.5
14	Methyl ethyl phenol	6.2
15	Methylethylphenol	2.6
16	2,3,5 -Trimethylphenol	4.7
17	Trimethylphenol	3.9
18	Trimethylphenol	10.4
19	Diethylphenol	0.3
20	Diethyl phenol	2.0
21	C6 substituted cyclohexane	1.9
22	C8 substituted cyclohexane	13.5
23	Cyclododecene	1.0
24	Ethenylundecene	1.7
25	Methyl -quinolfnone	1.4
26	Methyl -quinoline	2.8
	Total Resolved Hydrocarbons	453
	Total Unresolved Hydrocarbons	1015
	Total Hydrocarbons	1468
	Total Hydrocarbons F1 - F3	2339

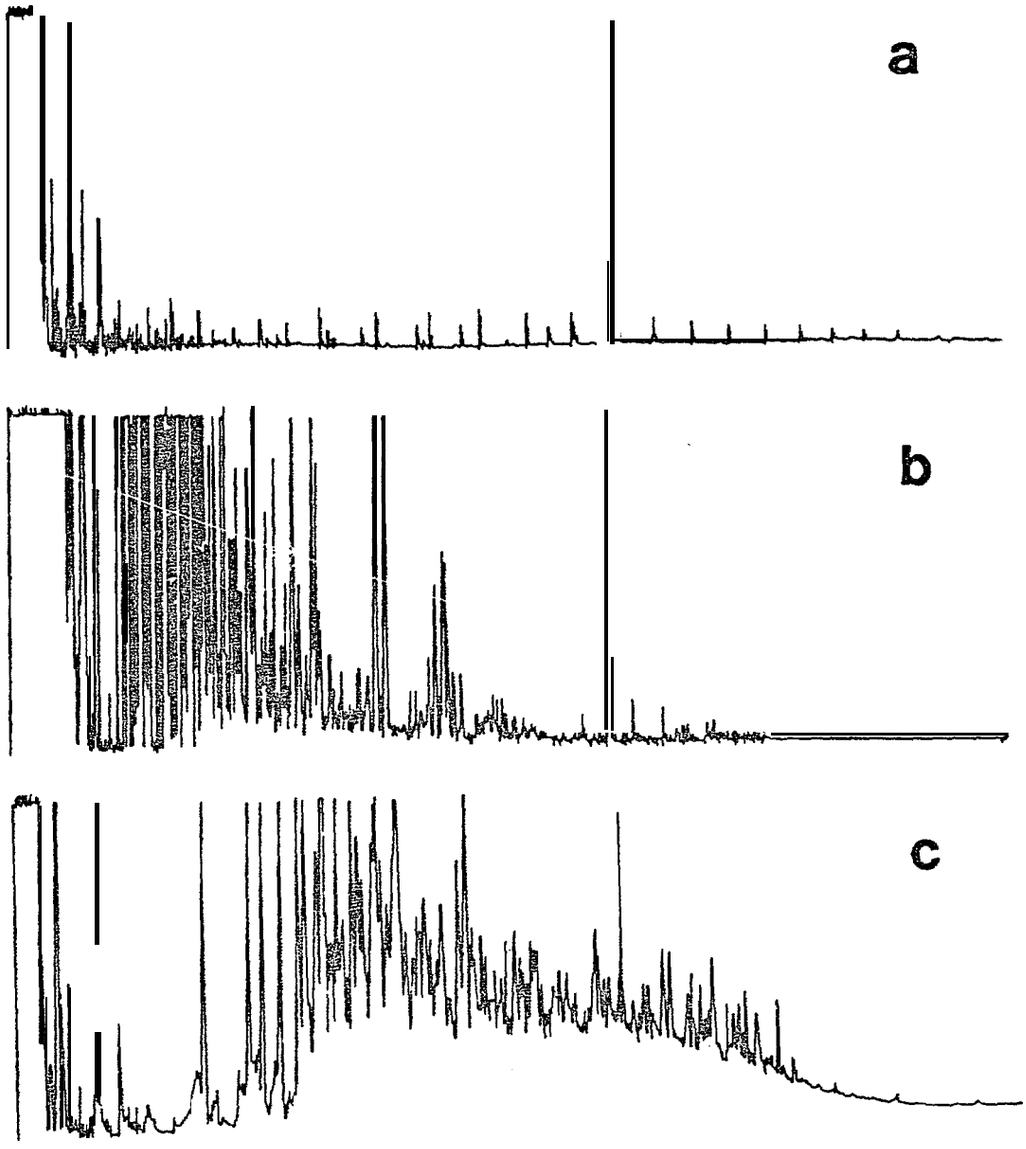


Figure II-12. Flame ionization detector capillary gas chromatograms of fractionated stock solution: a) aliphatic fraction: b) aromatic fraction; c) polar/heterocyclic fraction.

concentrations ranging from 1 to 33 µg/l. Additionally, a range of substituted naphthalenes were identified in the stock solution, including the parent compound and 6 methyl and dimethyl derivatives. Naphthalenes were observed to range in concentration from approximately 1 to 12 µg/l.

As evidenced by the capillary gas chromatogram (Figure II-11b) the lower molecular weight species were the dominant components in the aromatic fraction

of the aqueous stock solution. This is a result of the composition of the Santa Barbara crude, as well as the decreased aqueous volubility of the higher molecular weight aromatics. The composition of the aromatic water soluble fraction was generally very similar to that of the parental crude oil.

The quantity of polar/heterocyclic constituents, relative to aliphatic and aromatic components, was significantly greater in the aqueous test solution than that observed in the parental crude oil. As indicated in Table II-14, phenol and its alkyl-substituted homologs dominated the polar/heterocyclic fraction. Of the 26 compounds identified, 20 were members of the alkyl-substituted phenol family. This family of compounds not only accounted for the greatest number of species, but also the greatest individual and cumulative concentrations. Individual alkyl phenols were observed at concentration levels as high as 70 µg/l, and the cumulative concentration totaled 285 µg/l. The water soluble polar/heterocyclic components comprised over 50% of the total water soluble fraction.

In general, the crude solubilization process resulted in a complex aqueous test solution. As expected, the composition of the test solution was directly linked to the aqueous volubility of the individual organic species in the parental crude oil. Only a limited number of aliphatic hydrocarbons were observed, and of those identified none were present in excess of 1 µg/l. The composition of the aromatic water soluble fraction closely resembled that of the parental crude oil, and was dominated by alkyl-substituted benzenes and naphthalenes. Although very few polar/heterocyclic components were identified in the parental crude, the water soluble fraction contained substantial quantities of alkyl-substituted phenols. In general, low and intermediate molecular weight species dominated in all three fractions, due to the limited aqueous volubility of the higher molecular weight organics. Polar/heterocyclic components typically comprised 62.8% of the total water soluble fraction, whereas aromatic and aliphatic constituents comprised 36.6%% and 0.6%, respectively.

c. General WSF Characteristics

The composition of the low, medium and high WSF test solution concentrations closely paralleled that of the stock reservoir solution. Low levels of relatively few aliphatic/alicyclic hydrocarbons were observed in the WSF test solutions, and those were predominantly low molecular weight species. All WSF test solutions were dominated by the more soluble aromatic and polar/heterocyclic components. The lower molecular weight species (5 to 8 carbon atoms) were also the most abundant aromatic components identified. This was true both in terms-of the diversity of individual compounds, as well as their relative concentration levels. In addition to the lower molecular weight aromatics, largely alkyl -substituted benzenes, naphthalene and its alkyl-substituted homologs were routinely identified at significant concentration levels in the WSF test solutions. Other aromatic compounds identified in the WSF test solutions included substituted indenes, pyrans, and benzothiophenes.

The other category of compounds identified most frequently in the test solutions was the polar/heterocyclic group. This group of organics typically represented approximately 50% of the identifiable compounds. Phenol and its alkyl-substituted homologs dominated this class of compounds, which was typically characterized by C_1 through C_3 substituted species. Because of the greater aqueous volubility of phenolic compounds, individual concentration levels were generally high relative to other species. Substituted

furans, thiophenes and organic acids were also identified frequently in polar/heterocyclic extracts from aqueous test solutions over the course of the toxicity studies.

Figure II-13 presents a GC/MS reconstructed ion chromatogram from the analysis of the volatile fraction of a typical WSF test solution. This chromatogram is from the analysis of a high exposure level test solution collected from one of the larval experiments. A tabular summary of the components identified in this sample is presented in Table 11-15, with the numbers in parentheses corresponding to those of tne labeled chromatogram. Similarly, Figure II-14 presents capillary column gas chromatograms of the extractable fraction of representative test solution samples. A tabular summary of the components identified in the high exposure concentration, which corresponds to Figure II-14d, is given in Table 11-16. These test solution characterization data are representative of those observed over the course of both larval and adult experiments. Complete qualitative and quantitative characterization data for the extractable fraction of the four weekly test solution samples are presented in the data appendix.

An isomer of diethyltetrahydrofuran was identified consistently in WSF test solutions used in all experiments. This compound was observed at roughly comparable levels and consistent concentrations in each of the three exposure regimes and the control during all experiments. The mean concentration was 7.0+4.0 µg/l in the larval studies, and in adult studies the mean concentration was 8.3+3.7 µg/l. Although this compound occurred at a slightly lower mean concentration in control water from the larval and adult

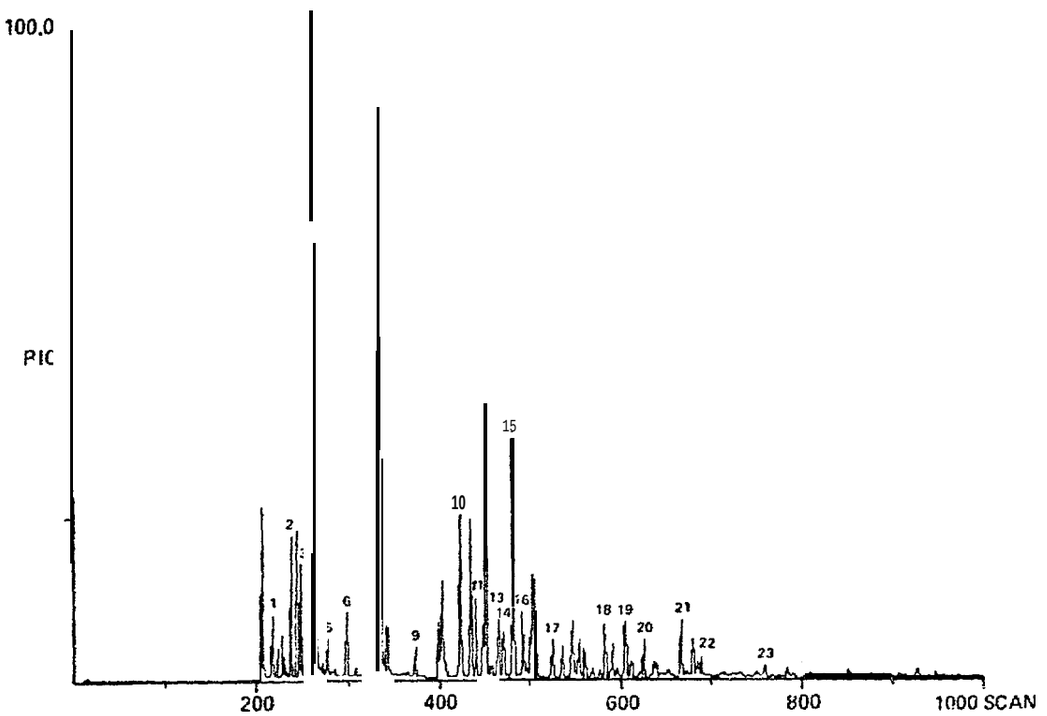


Figure II-13, GC/MS reconstructed ion chromatogram of the volatile fraction from a typical Mgh level test solution.

experiments, it was considered to be a system contaminant in both cases. The exact source of the contaminant was never definitively identified; however the adhesive used in the plumbing of the bioassay system seems a likely candidate. Phthalate esters, however, another group of commonly used" plasticizers, were rarely identified in the aqueous test solutions.

Table II-15. GC/MS identifications of selected components in the volatile fraction from a high exposure level test solution (see Figure H-13).

Peak Number	Compound	Conc. (mg/g)
1	Pentane	9.5
2	Hexane	17.9
3	Methylcyclopentane	20.1
4	Benzene	100.0
5	Methyl hexane	2.0
6	Methyl cyclohexane	7.4
7	Ethyl cyclopentane	*
8	Methyl benzene	87.3
9	Tetrahydrothiophene	4.4
10	Methyl-tetrahydrothiophene	21.5
11	Ethyl benzene	9.7
12	Dimethyl benzene	57.4
13	Methyl-tetrahydro-2H-thiopyran	2.1
14	Dimethyl-tetrahydrothiophene	11.8
15	Dimethylbenzene	42.8
16	Dimethyl-tetrahydrothiophene	9.2
17	Methyl-ethylbenzene	5.0
18	Trimethyl benzene	9.9
19	Methyl-ethylbenzene	6.6
20	Trimethyl benzene	5.2
21	Trimethyl benzene	10.7
22	Dihydro-1H-indene	3.7
23	C4 substituted benzene	*

* Concentration less than 0.1 ug/l

Thus, experimental organisms were exposed to the diethyltetrahydrofuran contaminant at roughly comparable concentrations during all bioassay experiments. Although all organisms were inadvertently exposed to this compound, it was not identified as a component of the primary toxicant, and as such is not considered to be a petroleum-dosed component. Since control organisms were also exposed to the diethyltetrahydrofuran, and the intent of this study was to evaluate the effect(s) of a dosed agent (the water-soluble fraction of Santa Barbara crude), a distinction has been made between the hydrocarbon dosing and exposure levels. Therefore, in subsequent sections, test solution hydrocarbon levels have been reported both with diethyl tetrahydrofuran included (the total exposure level) and without its inclusion (the total dosed hydrocarbon level). This distinction is essential for evaluation of the test solution exposure regime which is attributable to the water soluble fraction of the primary toxicant.

During the course of the adult northern anchovy experiment, the aqueous test solutions were rigorously monitored for a six day period. On each of the six days a single control sample and triplicate samples from the low, medium, and high concentration regimes were collected for analysis of both volatile and non-volatile hydrocarbon content. The purpose of this investigation was to evaluate the consistency and reproducibility of the physical dosing system, as well as the associated analytical variability. Although the level of volatile hydrocarbons was monitored daily in all experiments, extractable (non-volatile) hydrocarbon analyses were performed only once a week. For this reason, it was of interest to evaluate the physical dosing system with respect to non-volatile hydrocarbons by examining its profile on a daily basis. These data provided an additional means of assessing the utility of volatile analyses as an indicator of overall system performance. Study results (Table 11-17) were also used to evaluate the analytical variability associated with the two monitoring techniques that were employed during experiments.

d. Adult Hydrocarbon Tissue Burden Characterizations

One of the preliminary tasks conducted in conjunction with hydrocarbon tissue burden characterization was a series of spike/recovery studies on selected target tissue samples. These spike/recovery studies were conducted using selected petrogenic hydrocarbons to evaluate recovery efficiencies for the analytical protocol employed, and to establish detection limits

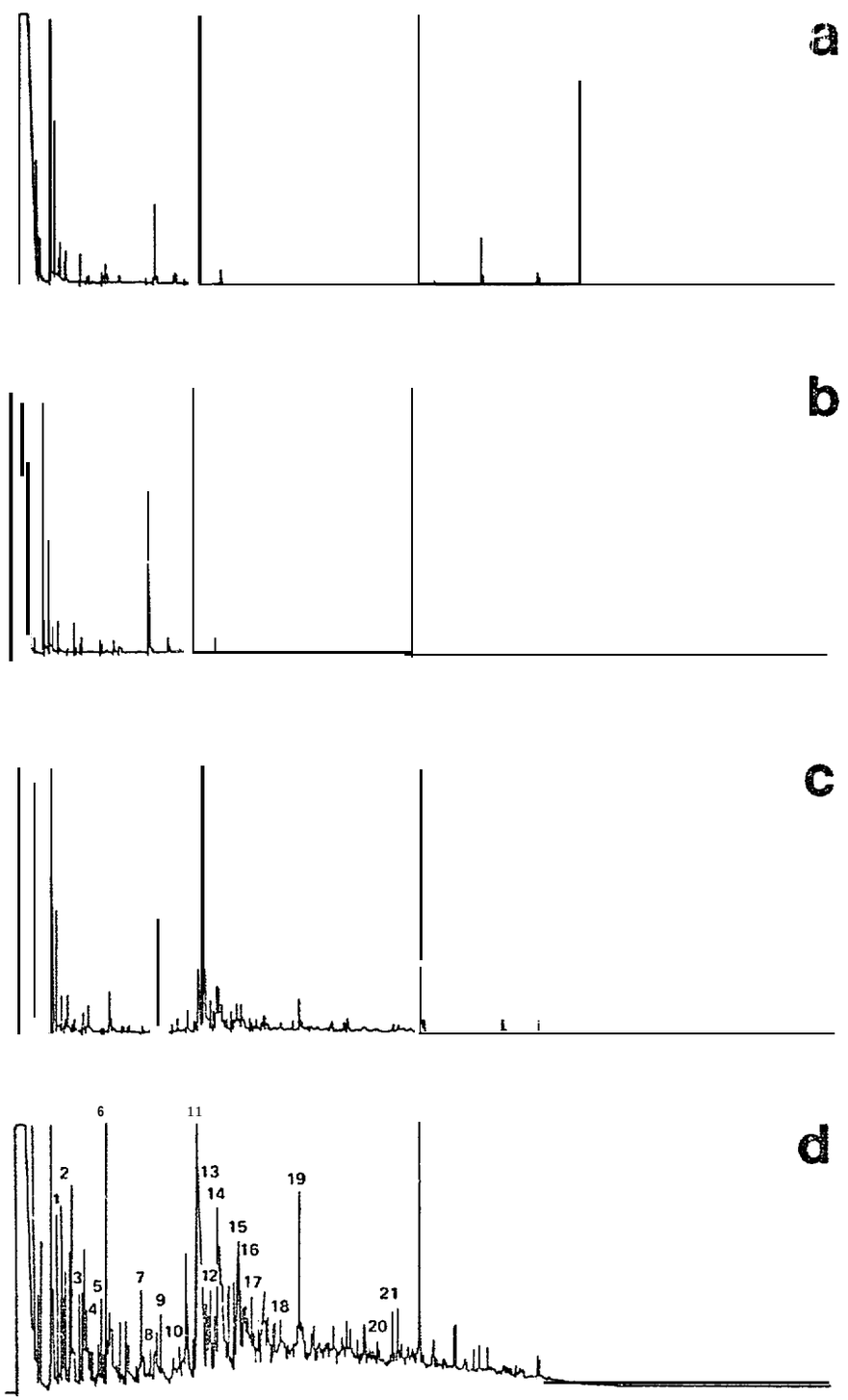


Figure II-14. Capillary column gas chromatogram 01 the extractable fraction horn typical test solutions a) control, b) low level, c) medium level, d) high level.

for the hydrocarbons of interest. Additionally, earlier spike /recovery studies were used to evaluate the proposed analytical protocol and make procedural modifications in order to optimize the accuracy and sensitivity of the technique.

Spike/recovery evaluations were performed on tissue samples from both California halibut and California mussel , using representative members of aliphatic, aromatic and polar/ heterocyclic petrogenic hydrocarbons. In all, 65 individual hydrocarbons were used in the spike/recovery investigations, and 7 to 10 replicate studies were conducted. Mixtures of individual compounds from each of the three groups of hydrocarbons were injected into the tissues in known quantities prior to any sample preparative procedures. The samples were then prepared and analyzed by the protocol employed in all subsequent tissue characterizations. Only aliquots from previously analyzed tissues were used in spike/recovery studies so that resultant data could be corrected for background tissue contributions.

Table 11-16 . GC/MS identifications of selected components in the extractable fraction from a high exposure level test solution (see Figure II-14a).

Peak Number	Compound	Conc. (mg/g)
1	p-Xylene	1.5
2	o,m-Xylene	1.3
3	Cumene	1.5
4	n-P ropyl benzene	0.5
5	Mesitylene	0.7
6	Phenol	6.0
7	2-Methyl phenol	1.4
8	4-Methyl phenol	0.8
9	2,6-Dimethylphenol	1.2
10	2,4-Dimethyl phenol	1.1
11	Diethyl tetrahydrofuran	6.0
12	3,4 - Dimethyphenol	*
13	C10 alkane	5.9
14	C6 thi ol	1.0
15	Trimethyl phenol	2.1
16	Trimethylphenol	0.8
17	Methyl -methyl ethyl phenol	1.9
18	C5 substituted cyclohexane	1.4
19	C6 substituted cyclohexane	3.1
20	C19 carboxylic acid ester	0.7
21	Ethenyl undecene	1.1
22	D-10 Phenanthrene (internal std.)	
	Total Resolved Hydrocarbons	122
	Total Unresolved Hydrocarbons	331
	Resolved/Unresolved Ratio	0.37
	Total Hydrocarbons	453

● Concentration less than 0.1 µg/l

Results of the spike/recovery studies are presented in Tables II-18 through 11-21. As evidenced by Table 11-18, the detection limit for individual hydrocarbons using the chosen analytical protocol averaged about 15 rig/g in each of the three groups of hydrocarbons. Detection limits are reported on a wet tissue weight basis, and are based on the use of 20-gram sample masses.

Recovery (Tables 11-19 through II-21 for aliphatic, aromatic and polar/heterocyclic hydrocarbon groups, respectively) for Individual hydrocarbons was very consistent within each hydrocarbon group. This is significant because the individual compounds utilized are representative of the full spectrum of hydrocarbons encountered in whole crude oils; and include low, intermediate and high molecular weight species. As these data demonstrate, recoveries averaged about 57% for aliphatics, 47% for aromatics and 24% for polar/heterocyclic hydrocarbons.

It is particularly important to bear these data in mind, because hydrocarbon tissue burden data reported in subsequent sections are uncorrected for analytical recovery efficiency. This approach was selected because recoveries could not be determined for the myriad of hydrocarbons potentially encountered in petroleum e. posed organisms, and therefore exact corrections could not always be calculated. However, it should be realized that data generated during spike/recovery studies suggest that recoveries are relatively consistent within a given group of hydrocarbons, and that the average recoveries developed provide a means of more closely estimating actual hydrocarbon levels, Thus, for a typical aromatic compound the actual (best estimate)

Table II-17. Data summary from physical dosing system/analytical variability study.

Collection Date	Exposure Level (Hydrocarbon Concentration in µg/l)[a] Control VOA[b]	Control Ext.	Control Total	Low VOA	Low Ext.	Low Total	Medium VOA	Medium Ext	Medium Total	High VOA	High Ext.	High Total
9/27/82	0.3	6.0	6.3	4.9 ±0.9	5.8 ±1.0	10.8 ±1.3	20.3 ±2.8	6.9 ±0.7	27.2 ±3.1	189 ±3	24.6 ±0.3	214 ±3
9/28/82	0.2	9.8	10.0	2.1 ±0.1	10.8 ±0.7	12.9 ±0.7	22.1 ±0.9	12.7 ±1.2	34.8 ±1.7	163 ±7	34.0 ±5.5	197 ±13
9/29/82	0.1	10.7	10.8	1.4 ±0.5	14.7 ±2.3	16.1 ±2.7	24.3 ±2.5	17.9 ±0.1	42.3 ±2.4	151 ±6	44.4 ±9.6	195 ±11
9/30/82	0.2	11.0	11.2	-2.0 ±0.6	13.5 ±1.1	15.5 ±1.7	25.0 ±2.2	19.9 ±1.7	44.8 ±3.0	188 ±4	34 .5 ±2.5	222 +6
10/1/82	0.1	16.1	16.2	2.8 ±0.2	74.7 ±2.9	17.5 +2.9	32.2 ±3.8	17.3 ±5.4	49.6 +9.2	230 ±12	48.9 ±4.1	279 ±9
10/2/82	0.2	14.9	15.1	4.9 ±0.7	14.9 +3.0	19.8 +3.1	31.4 ±1 .4	19.7/ ±2.0	51.1 +0.8	193 ±2	40.7 ±2.7	240 +5
6 Day Summary	0.2 +0.1	11.4 ±3.7	11.6 +3.6	3.0 ±1.5	12.4 ±3.8	15.4 +3.6	25.9′ ±5.1	15.8 ±5.2	41.6 ±9.3	187 ±27	37.1 ±9.4	225 ±31

a With the exception of control values, alldaily data are means +1 standard deviation for triplicate analyses.

b VOA = Volatile organic fracti On.

tissue burden value would be approximately twice the reported value since the mean recovery for aromatics was observed to be about 50%.

Several considerations should also be noted relative to the tissue hydrocarbon burden characterizations. The analytical protocol employed for tissue analyses was focused on characterization of semivolatile and non-volatile hydrocarbons. Although a few very volatile hydrocarbons are not amenable to characterization with this approach, selection of protocol was based on its compatibility with the broadest spectrum of potential hydrocarbon contaminants. The limitations imposed by the sample extraction and preparation technique used are confined to the inability to characterize hydrocarbon contaminants containing less than seven carbon atoms. This limitation is not of great consequence however, because the parent toxicant contained relatively few compounds in this size range. Additionally, it should be noted that quantification of some semi-volatile components (7 and 8 carbon atoms) may not be as accurate as that for less volatile compounds.

Table II-18. Detection limits for selected petrogenic hydrocarbons in biological tissues(a) (ng/g).

Hydrocarbon Category	Number Of Individual Compounds Evaluated[b]	Detection Limit Data[c] Range	Mean	Standard Deviation
Aliphatic (F1)	26	10-30	16	6
Aromatic (F2)	23	9-20	14	2
Polar /Heterocyclic (F3)	11	13-17	15	1

a Data based on spike/recovery studies utilizing California halibut (Paralichthys californicus) muscle tissue and whole California mussel (Mytilus californianus) organisms.

b Individual compounds correspond to those given in the spike/recovery results tables.

c Detection limit data based on analysis of 20 gram (wet weight) tissue masses.

Most of the hydrocarbon tissue burden results are focused on examination of the aromatic (F2) fraction. The selection of the aromatic fraction as a focal point is a result of the nature of the primary toxicant, the fractionation scheme employed and the ability to distinguish petrogenic hydrocarbons from biogenic hydrocarbons. Over the course of the experimentation, it became evident that the aliphatic (F1) and polar/heterocyclic (F3) tissue extract fractions were of limited utility in evaluating the degree of petroleum contamination. For aliphatic hydrocarbons, the limitations are due to two factors. Although the primary

Table II-19. Spike/recovery data for aliphatic hydrocarbons in biological tissues(a).

Spiked Compound	Spike[b] Level (ng/g)	Numbers of Replicates	Mean % Recovery	Standard Deviation % Recovery
nC-9	36	9	71.8	11.8
nC-10	37	7	28.5	10.3
nC-11	37	7	40.7	12.9
nC-12	69	10	44.9	22.9
nC-13	6.2	10	32.8	27.5
nC-14	61	-9	49.0	22.4
nC-15	67	8	543.9	23.5
nC-16	6s	9	55.6	11.4
nC-17	66	9	69.1	21.4
Pri stane	65	9	66.1	17.2
nC-1 8	65	9	55.5	12.2
Phytane	63	10	53.4	20.3
nC-19	64	9	54.5	17.6
nC-20	65	10	64.8	26.7
nC-21	66	10	62.2	32.2
nC-22	58	9	61.8	37.0
nC-23	64	10	56.4	33.4
nC-24	63	9	59.4	27.3
nC-25	65	8	50.9	25.0
nC-26	64	9	62.9	29.7
nC-27	64	7	70.0	44.5
nC-28	66	10	67.0	26.6
nC-29	62	10	62.7	20.9
nC-30	64	9	56.5	29.3
nC-31	63	8	60.0	38.2
nC-32	65	9	57.6	36.2
Summary (all compounds)		224	56.6	10.7

[a] Data are a composite from spike/recovery studies of California halibut (Paralichthys californicus) muscle tissue and whole California mussel (Mytilus californianus) organisms.

[b] Spike level on wet weight basis.

crude oil toxicant contained a variety of aliphatic hydrocarbons, these compounds were of limited aqueous volubility and thus were present at low levels in test solutions relative to aromatic and polar/heterocyclic components. More importantly, aliphatic hydrocarbons of petrogenic origin are very difficult to distinguish from those of biogenic origin because many of the individual compounds are common to both systems.

Polar/heterocyclic (F3) tissue extracts were of limited value because the primary crude toxicant contained relatively few compounds of this type and many biogenic components are members of this category of organic compounds. Although significant levels of phenolic hydrocarbons appeared in the WSF test solutions, these compounds did not accumulate at detectable levels in the tissue extracts. Accumulation of other polar/heterocyclic contaminants was generally

Table II-20. Spike/recovery data for aromatic hydrocarbons in biological tissues(a).

Spiked Compound	Spike[b] Level (ng/g)	Numbers of Replicates	Mean % Recovery	Standard Deviation % Recovery
Ethyl benzene	101	7	20.6	9.6
p-Xylene	99	7	61.7	28.5
o,m-Xylene	97	7	25.6	9.9
Cumene	102	10	22.0	7.8
n-Propylbenzene	99	10	22.1	11.9
Mesitylene	101	10	30.4	19.2
p-Cymene	97	10	19.6	9.4
n-Butyl benzene	100	10	25.0	8.7
Naphthalene	100	10	44.1	16.0
Benzothiophene	100	10	52.7	13.8
n-Hexylbenzene	99	10	38.1	12.3
2-Methyl-naphthalene	100	10	48.9	15.0
1-Methyl-naphthalene	100	10	49.3	14.5
Biphenyl	100	10	53.2	12.4
2,6-Dimethyl-naphthalene	100	10	61.1	24.6
n-Octylbenzene	100	10	52.2	13.1
2,3,5-Trimethyl-naphthalene	100	10	57.6	10.8
Fluorene	100	10	57.2	11.6
Dibenzothiophene	100	10	54,5	8.3
Phenanthrene	100	4	55.4	9.3
Anthracene	100	10	60.1	20.8
1-Methylphen-anthrene	100	10	59.9	9.8
Fluoranthene	75	10	62 3	12.6
Pyrene	100	10	60.1	13,4
Benzo(b)-anthracene	100	10	58.1	16.7
Chrysene	100	10	55.9	16.5
Benzo(e)pyrene	75	10	51.6	18.7
Perylene	100	10	56.2	24. 8
Summary (all compounds)		265	47.0	14. 7

[a] Data are a composite from spike/recovery studies of California halibut (Paralichthys californicus) muscle tissue and whole California mussel (Mytilus californianus) organisms.

[b] Spike level on wet weight basis.

Table II-21. Spike/recovery data for polar hydrocarbons in biological tissues(a).

Spiked Compound	Spike[b] Level (ng/g)	Numbers of Replicates	Mean % Recovery	Standard Deviation % Recovery
Phenol	780	7	16.1	8.1
2-Methylphenol	788	7	22.5	6.3
4-Methylphenol	780	7	25.0	6.9
2,6-Dimethylphenol	773	7	21.7	5,6
2,4-Dimethyl phenol	780	7	25.6	6.9
3,5-Dimethylphenol	729	7	28.9	7.1
2,3-Dimethyl phenol	720	7	24.7	6.9
3,4-Dimethylphenol	810	7	29. S	7.1
2,4,6-Trimethyl phenol	747	7	21.5	4.7
2,3,4-Trimethyl-phenol	578	7	24.5	5.3
2,3,5-Trimethyl-phenol	720	7	26.9	11.0
Summary (all compounds)		77	24.3	3.6

[a] Data are a composite from spike/recovery studies of California halibut (Paralichthys californicus) muscle tissue and whole California mussel (Mytilus californianus) organisms.

[b] Spike level on wet weight basis.

obscured by the massive quantity of polar biogenic material. Also, several of the more important groups of heterocyclic compounds, such as thiophenes, furans and pyrans, routinely appeared in the aromatic tissue extract fraction (F2).

As a result of these phenomena, the aromatic tissue extract fractions were found to contain the vast majority of identifiable petrogenic hydrocarbons. Examination of this extract fraction was also important since the petroleum constituents of greatest toxicological consequence are generally limited to this generic category of organics. Based on results from the halibut, mussel and initial anchovy tissue characterizations, only aromatic tissue extracts were characterized in the adult anchovy experiment.

quantitative data on biogenic hydrocarbons from tissue extracts should be considered rough approximations. These data, which are found in the detailed characterizations presented in the data appendix, should only be used for relative comparison and not as absolute values. Because the emphasis of the analytical work was on identification and quantification of petrogenic hydrocarbons, sample preparation procedures often resulted in excluding all or part of various biogenic hydrocarbon groups. Although the reported data are representative of tissue extracts as processed, they are not necessarily representative of the biogenic hydrocarbon composition of the tissues. Additionally, many biogenic hydrocarbons are not accurately quantified by the analytical protocol employed because of their high molecular weight, the large quantities of material present, and/or inherent chemical properties.

2. CALIFORNIA HALIBUT (Paralichthys californicus)

a. Embryo Experiment

1) Hydrocarbon Exposure. Hydrocarbon exposure data for the California halibut embryo experiment are summarized in Table II-22. High background hydrocarbon levels resulted in statistically indistinguishable differences between control and low exposure level hydrocarbon concentrations. Corrected control and low exposure level hydrocarbons concentrations were in the range of 6-IO µg/l.

Table II-22. Hydrocarbon exposure concentrations for California halibut embryo experiment.

Exposure Level	Hydrocarbon Concentration (µg/l)[a] Volatile Fraction	Extractable Fraction	Total
Control	0.8	5.0 [13.2]	5.8 [14.0]
Low	3.5	6.7 [11.9]	10.2 [15.4]
Medium	68.5	22.7 [32.4]	91.3 [101]
High	517	249 [264]	761 [7?61

[a] Extractable and total hydrocarbon values are reported corrected for the presence of diethyltetrahydrofuran and uncorrected values are also presented in brackets.

Total hydrocarbon concentration levels were 101 and 776 µg/l for medium and high level test solutions, respectively. Anomalous organic compounds were not detected in any of the test solutions analyzed.

2) Hydrocarbon Tissue Burden. Hydrocarbon bioaccumulation by P. californicus embryos exposed to the three WSF test solution concentrations was indirectly measured by radioactive tracer (i.e. ^{14}C-naphthalene) uptake. Mean radioactive tracer uptake measured from newly-hatched larvae was directly proportional to the WSF exposure concentration (Table II-23). For example, mean radioactive tracer uptake per embryo was 16.0 cpm for embryos exposed to the low concentration and approximately 17 times higher (267.7 cpm) for embryos in the high concentration. Control embryos exhibited only a negligible uptake of radioactive tracer. A K-W ANOVA demonstrated that tracer

Table II-23. Mean radioactive tracer uptake per larvae, mean percent mortality, mean percent hatching success, mean total length of newly-hatched, and mean percent malformed newly-hatched California halibut larvae following 72 hours of embryonic lest solution exposure, Standard errors are **shown in parentheses.**

Concentration	Mean Radio-active Tracer uptakea (cpm)	Mean % Embryo Mortal i t,a	K-can % Hatching Success[a]	Mean Total Length of Newly-Hatched Larvae[a] (mm)	Mean Percent Mal formed Newly-Hatched Larvae[a]
Cent rol	2.8[b] (0.24)	13.3[b] (2.78)	86.7[b] (2.82)	2.29[b] (0.02)	1.9[c] (0.49)
Low	16.0[b] (2.90)	16.4[b] (2.40)	83.2[b] [2.09)	2.34[b] (0.04)	5.7[b] (0.98)
Medium	85.2[c] (15.70}	22. 0[c] (2.33]	78. 0[c] (2.33)	2.33[b] (0.03)	6.7[b] (1.18)
High	267.7[d] (49.87)	29. 0[d] (2.78)	71. 0[c] (2.74)	1.99[c] (0.45)	20.6[d] (2.66)

[a] Significant difference (P <0.05) among groups; K-W ANOVA.

b,c,d Groups designated by different letters significantly different (p <0.05) from all other groups; nonparametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

uptake was significantly different among WSF-exposed and control embryos (p <0.05). Results of multiple comparison testing indicated that embryo tracer uptake was: 1) significantly greater in the high concentration than in the control, low, or medium concentrations; and 2) significantly greater in the medium concentration than in either the low concentration or control. Uptake by embryos exposed to the low concentration was not significantly different from control embryos.

3) Embryo Mortality. Mean mortality of P. californicus embryos per replicate ranged from 16.4 to 29.0% in the low and high concentrations, respectively, and increased with exposure to increasing WSF concentrations (Table II-23; data appendix). A K-W ANOVA based on ranked data indicated that mortality was significantly different (p <0.05) among WSF-exposed and control embryos. Multiple comparison testing indicated no significant difference in embryo mortality between the control and low concentration, but that exposure to both the medium and high concentrations resulted in significantly elevated embryo mortality compared with controls. Embryo mortality was also significantly greater in the high concentration than in the medium concentration.

Embryo hatching success decreased with exposure to increasing test solution concentrations, ranging from 86.7% in the control to 71.0% in the high concentration (Table II-23). A K-W ANOVA showed that hatching success was significantly different among WSF-exposed and control embryos (p <0.05). Multiple comparison testing demonstrated: 1) no significant difference in hatching success between the control and low concentration; 2) significantly depressed hatching success at the medium and high test solution concentrations; and 3) no significant difference in embryo hatching success between the medium and high test solution concentrations.

4) Development. Development of newly-hatched larvae from WSF-exposed and control embryos was primarily assessed by examining the size (i.e. total length) of larvae and the incidence of larvae with bent notochords. In addition, microscopic morphometric measurements were made for the following characters: snout-vent length, yolk sac diameter, eye diameter, otic vesicle diameter, and the distance from the eye to tip of snout.

The mean total length of newly-hatched larvae at hatching ranged from 1.99 to 2.34 mm in the high and low concentrations, respectively (Table II-23). Control larvae were generally comparable in size to those hatching from embryos exposed to the low and medium concentration exposure regimes. A K-W ANOVA and subsequent multiple comparison testing demonstrated that size at hatching was significantly lower in the high Concentration than in the control, low, or medium concentrations.

The incidence (i.e. mean percent occurrence) of larvae with bent notochords hatching from WSF exposed embryos ranged between 5.7% (low concentration) and 20.6% (high concentration), respectively (Table 11-23). In contrast, the incidence of bent notochords among larvae hatching from control embryos was only 1.9% (Table II-23). Results of a K-W ANOVA and subsequent multiple comparison testing demonstrated: 1) the incidence of bent notochords was significantly greater for larvae hatched from WSF exposed embryos than for larvae hatched from control embryos; 2) there was no significant difference in the incidence of bent notochords occurring in larvae hatched from low and medium concentration exposed embryos; and 3) the incidence of bent notochords in larvae hatched from embryos exposed to the high concentration was significantly greater than for larvae hatched from embryos held in the low and medium concentration exposure regimes.

5) Histopathology Histological examination of selected organs in larvae was not feasible during the program, since an extensive research and development effort was needed. However, whole organism mounts were prepared and subjected to microscopic examination. These results were presented in Section 4.a.4), Development.

Morphometry of newly-hatched larvae indicated that only snout-vent (S-V) length and eye diameter were significantly different for larvae exposed to WSF (Table II-24). S-V lengths of larvae from the medium and high concentration exposure regimes were significantly reduced compared with control larvae (Plate II-la-d). There was no significant difference between the S-V length of larvae from either the control and low or the medium and high concentrations, respectively (Table 11-24). Differences in S-V length were generally correlated with differences in total larval length. Similarly, the eye diameter of newly-hatched larvae from all WSF exposure regimes was significantly reduced in size compared with control larvae (Plate Ii-la-d). No significant differences in yolk sac diameter, otic vesicle diameter or eye-snout distance were found between larvae hatched from control or WSF-exposed embryos (Table 11-24).

Table II-24. Mean morphometric measurements of newly-hatched larval P. californicus from the embryo experiment, Standard errors in parentheses.

Morphometric Measurement (mm)[a]	Concentration			
	Control	Low	Medium	High
Snout-vent length[b]	1.35[c] (0.04)	1.34[c] (0.04)	1.18[d] (0.03)	1.17[d] (0.05)
Yolk-we: Sum of anterior-posterior and dorsal-ventral diameters[f]	1.28 (0.02)	1.30 (0.02)	1.31 (0.03)	1.31 (0.02)
Diameter of Eye: Anterior-posterior[b]	0.22[c] (0.004)	0.20[e] (0.004)	0.20[e] (0.004)	0.18[d] (0.004)
Diameter of Otic Vesicle: Anterior-posterior[f]	0.05 (0.004)	0.05 (0.004)	0.05 (0.004)	0.05 (0.004)
Distance from Eye to Tip of Snout[f]	0.07 (0.004)	0.07 (0.004)	0.07 (0.004)	0.07 (0.004)

[a] Based on measurements of six larvae.

[b] Significant difference among groups (p <0.05); K-W ANOVA.

[c, d, e] Groups designated by different letters Significantly different (p <0.05) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

[f] No signficant difference among groups; K-W ANOVA.

b. Larval Experiment

1) Hydrocarbon Exposure. Hydrocarbon exposure data for the larval

California halibut (Paralichthys californicus) experiment are summarized in Table II-25). High background hydrocarbon levels resulted in statistically indistinguishable differences between control and low exposure level hydrocarbon concentrations. Total corrected control and low exposure level hydrocarbon concentrations were in the range of 8 to 10 µg/l. Mean total hydrocarbon values for the medium and high concentration exposure regimes were 69.5 µg/l and 606 µg/l, respectively.

2) Hydrocarbon Tissue Burden. Radioactive tracer uptake was used as an indirect measure of hydrocarbon bioaccumulation by larval P. californicus. Tracer uptake measurements were performed on larvae from replicates terminated on days 3, 7, 14, and 18 of the experiment (Table II-26; Figure II-15; data appendix).

Following three days of exposure, mean tracer uptake by larvae in the medium concentration was elevated by a factor of nearly 60 times compared with that of the control and low concentration exposure regimes (Table II-26 and Figure 11-15). Because of extremely high mortality in the high concentration regime by day 3, only a single estimate of tracer uptake could be obtained. Comparison of this single estimate with larval uptake in the control and low concentrations indicated that uptake was elevated by more than a factor of 12 times. A K-W ANOVA based on ranked uptake data demonstrated that tracer uptake by medium concentration larvae was significantly ($p < 0.05$) greater than in either the control or low concentration regime larvae. No significant difference in tracer uptake was found between low concentration and control larvae.

Radioactive tracer uptake on day 7 was determined for three replicates from the control and low concentration, and for two replicates from the medium concentration. After 7 days of test solution exposure, tracer uptake was further elevated in medium concentration larvae, yet there was little evidence of additional uptake by larvae in the control or low concentration (Table II-26 and Figure II-15). A K-W ANOVA showed that larval tracer uptake continued to be significantly elevated in the medium concentration when compared with larval uptake in the

Table II-25. Hydrocarbon exposure summary for California halibut larval experiment.

Exposure Level	Hydrocarbon Concentration (µg/l)[a] Volatile Fraction	Extractable Fraction[b]	Total[c]
Control	0.6: 0.5	8.0± 4.2 [16.4± 4.5]	8.8± 4.0 [17.2± 4.31
Low	4.2± 2.4	6.3± 0.6 [12.4± 0.7]	10.5± 1.1 [16.7± 0.3]
Medium	50.4±30.4	19.1± 5.7 [25.0±10.5]	69.5±20.2 [75.4±25.5]
High	357±117	249 [264]	606 [6211

a Where appropriate, data are reported as the mean ±1 standard deviation.

b Extractable hydrocarbon values are reported corrected for the presence of diethyl tetrahydrofuran and uncorrected values are presented in brackets.

c Total hydrocarbon values represent the sum of the average daily volatile fraction plus the weekly extractable fraction. Values are reported both corrected and uncorrected for diethyl tetrahydrofuran.

Table H-26. Mean radioactive tracer uptake (counts per larvae per minute) by Paralichthys californicus larvae following 3, 7, 14, and 18 days of exposure, Standard errors are shown in parentheses.

Concentration	Exposure Period (Days) 3a	7a	14[b]	18[b]
Control	5.56[c] [0.19)	6.54[c] (0.80)	21.43 (14.48)	21.8 (0.86)
Low	5.65[c] (0.36)	7.59[c] (0.63)	44.67 (7.41	249.6 (210.75)
Medium	182.71[d] (26.5)	1004.15[d] [140.91)	---	---
High	124.68 (---)	---	---	---

a Tracer uptake significantly different ($p < 0.05$) among groups; K-W ANOVA.

b Tracer uptake significantly different ($p < 0.05$) among groups; one-tailed Mann-Whitney test.

c,d Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

The mean total length of newly-hatched larvae at hatching ranged from 1.99 to 2.34 mm in the high and low concentrations, respectively (Table II-23). Control larvae were generally comparable in size to those hatching from embryos exposed to the low and medium concentration exposure regimes. A K-W ANOVA and subsequent multiple comparison testing demonstrated that size at hatching was significantly lower in the high concentration than in the control, low, or medium concentrations.

The incidence (i.e. mean percent occurrence) of larvae with bent notochords hatching from WSF exposed embryos ranged between 5.7% (low concentration) and 20.6% (high concentration), respectively (Table II-23). In contrast, the incidence of bent notochords among larvae hatching from control embryos was only 1.9% (Table II-23). Results of a K-W ANOVA and subsequent multiple comparison testing demonstrated: 1) the incidence of bent notochords was significantly greater for larvae hatched from WSF exposed embryos than for larvae hatched from control embryos; 2) there was no significant difference in the incidence of bent notochords occurring in larvae hatched from low and medium concentration exposed embryos; and 3) the incidence of bent notochords in larvae hatched from embryos exposed to the high concentration was significantly greater than for larvae hatched from embryos held in the low and medium concentration exposure regimes.

5) Histopathology. Histological examination of selected organs in larvae was not feasible during the program, since an extensive research and development effort was needed. However, whole organism mounts were prepared and subjected to microscopic examination. These results were presented in Section 4.a.4), Development.

Morphometry of newly-hatched larvae indicated that only snout-vent (S-V) length and eye diameter were significantly different for larvae exposed to WSF (Table 11-24). S-V lengths of larvae from the medium and high concentration exposure regimes were significantly reduced compared with control larvae (Plate II-la-d). There was no significant difference between the S-V length of larvae from either the control and low or the medium and high concentrations, respectively (Table II-24). Differences in S-V length were generally correlated with differences in total larval length. Similarly, the eye diameter of newly-hatched larvae from all WSF exposure regimes was significantly reduced in size compared with control larvae (Plate II-la-d). No significant differences in yolk sac diameter, otic vesicle diameter or eye-snout distance were found between larvae hatched from control or WSF-exposed embryos (Table I I-24).

Table II-24. Mean morphometric measurements of newly-hatched larval P. californicus from the embryo experiment. Standard errors in parentheses.

Morphometric Measurement (mm)[a]	Concentration			
	Control	Low	Medium	High
Snout-vent length[b]	1.35[c] (0.04)	1.34[c] (0.04)	1.18[d] (0.03)	1.17[d] (0.05)
Yolk-sac: Sum of anterior-posterior and dorsal -ventral diameters[f]	1.28 (0.02)	1.30 (0.02)	1.31 (0.03)	1.31 (0.02)
Diameter of fye: Anterior-posteri or[b]	0.22[c] (0.004)	0.20[e] (0.004)	0.20[e] (0.004)	0. 18[d] ((). 004)
Diameter of Otic Vesicle: Anterior-posterior[f]	0.05 (0.004)	0.05 (0.004)	0.05 (0.004)	0.05 (0.004)
Distance from Eye to TIP of Snout[f]	0.07 (0.004)	0.07 (0.0041	0.07 (0.004)	{0.004)

[a] Based on measurements of six larvae.

[b] Signi ficant di f ference among groups (p ~0.051; K-W ANOVA.

[c,d,e] Groups designated by different letters significantly different (p <0. 05) from al l other groups; non-parametric analog to SNK multi ple comparison test. Groups with same letter not significantly different.

[f] No signficant di fference among grouos; K-W ANOVA.

b. Larval Experiment

1) Hydrocarbon Exposure. Hydrocarbon exposure data for the larval

Plate II-1(a-d). Representative photographs of newly hatched whole mounted larvae of California halibut from control group (a) and low (b), medium (c), and high (d) concentration exposed embryos. Structures in photographs are designated by uppercase letters as follows: eye (E), fin (F), otic vesicle (0), vent (V), yolk (Y). All larvae are magnified 43X.

a-b. Control, low, medium: Consult text and Tables II-23 and II-24 for significance of morphometric differences between all groups.

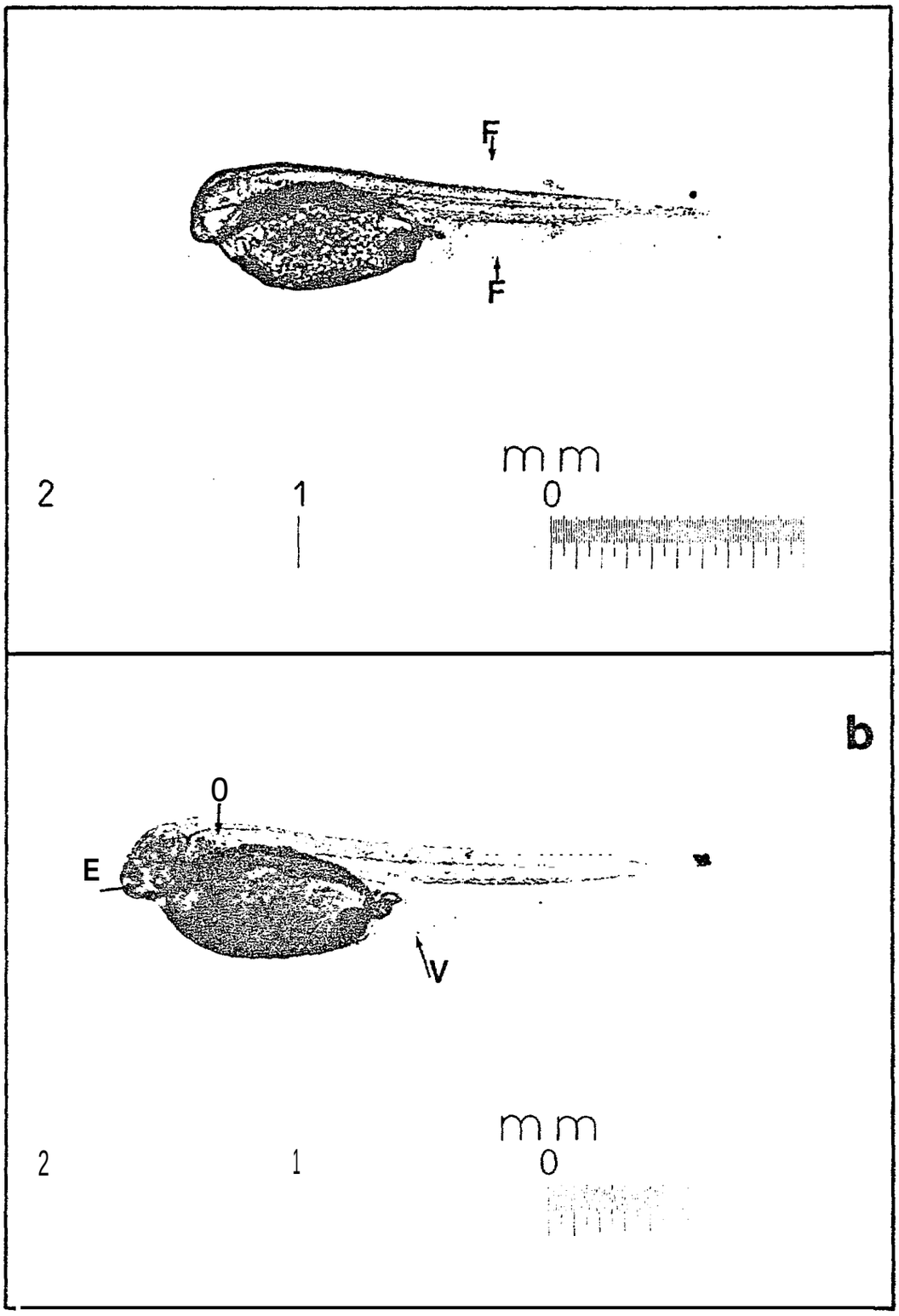

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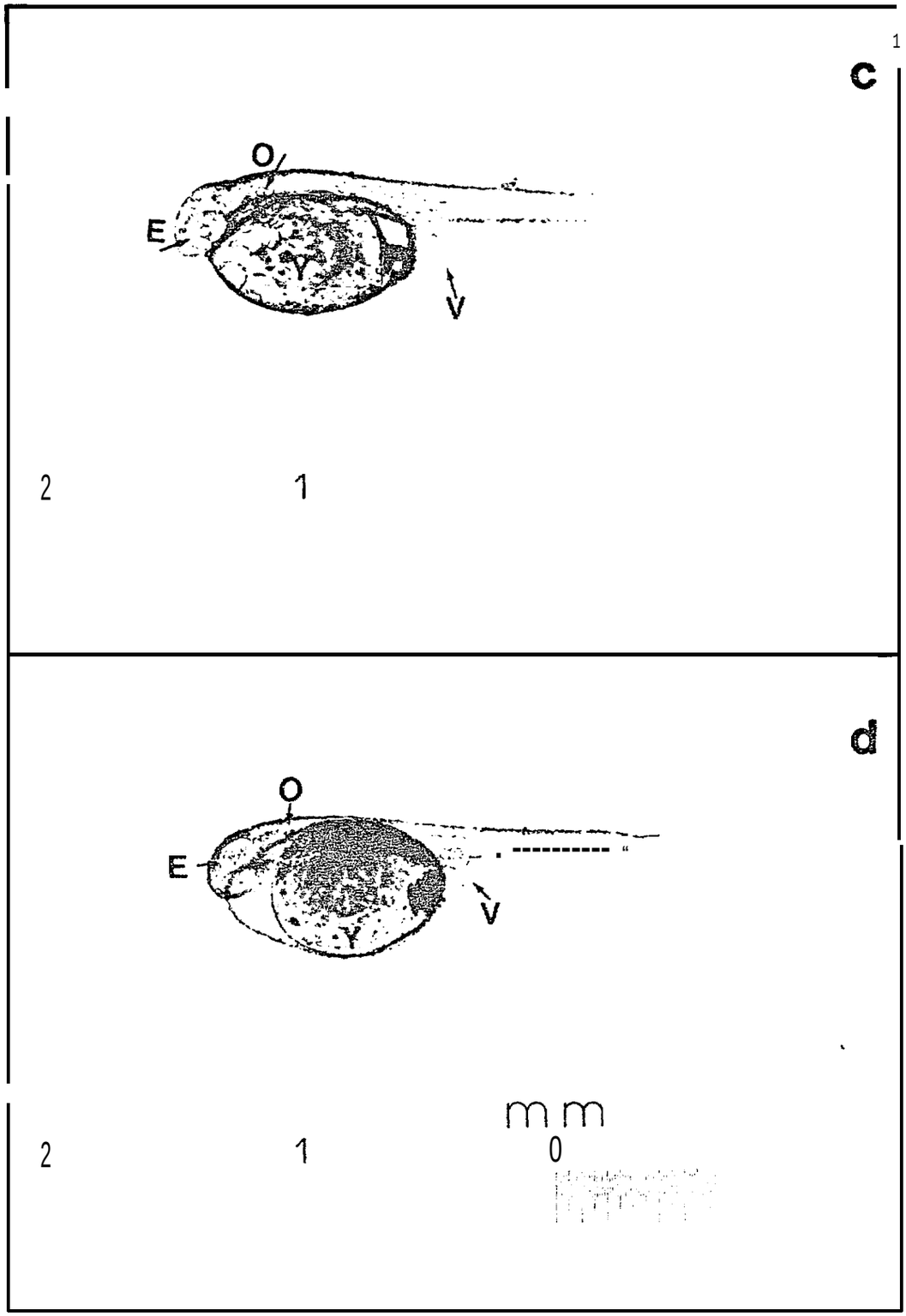
Plate II-1(a-d). (Cont)

c. Control, low, medium: Consult text and Tables II-23 and II-24 for significance of morphometric differences between all groups.

d. High: Larva is significantly shorter in total and snout vent length, and the eye is of lesser diameter than in the other three groups. The head is poorly formed. Note lens faintly visible in eye (E) and two otoliths in otic capsule (0).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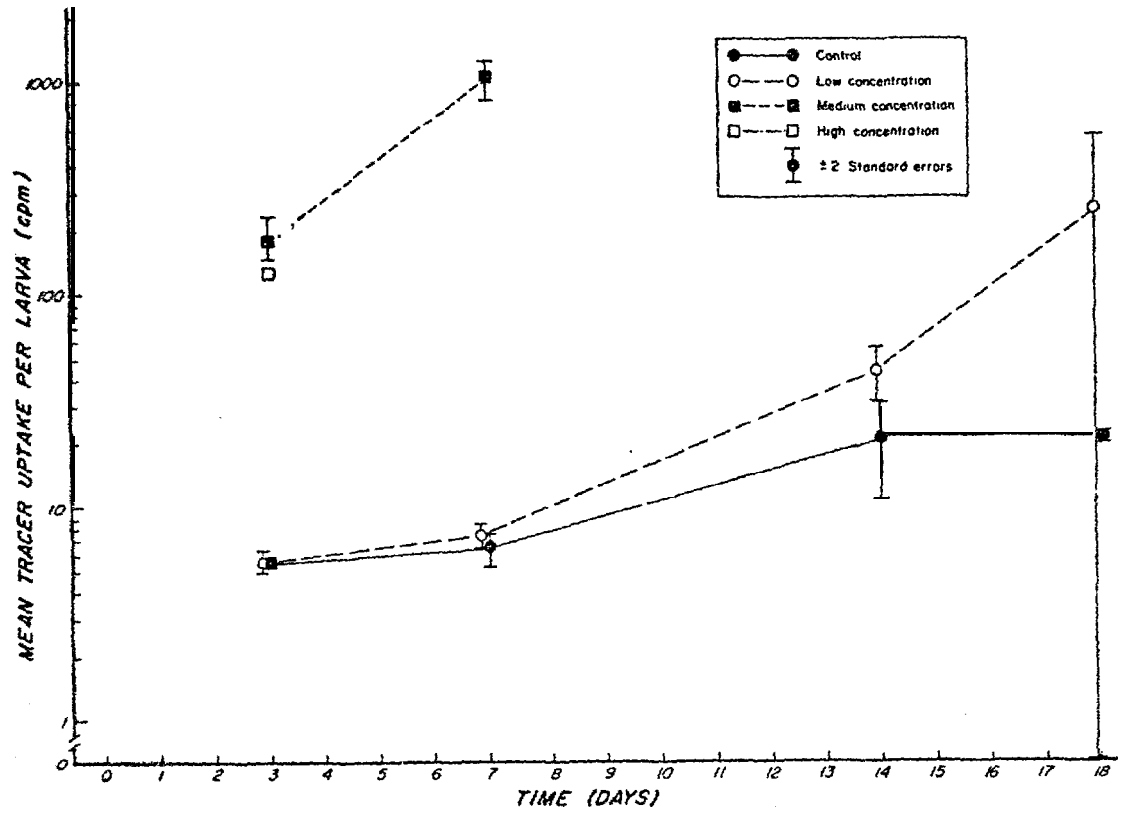

Figure II-15. Mean radioactive tracer uptake by larva} California halibut following 3,7,14, and 18 days of test solution exposure.

control or low concentration, No significant difference in tracer uptake was demonstrable between low concentration and control larvae after 7 days,

After 14 days of exposure, larval tracer uptake was determined for three replicates from the control and low concentration. The medium concentration was terminated prior to day 14 of the experiment because of high mortality, and no larvae were available for tracer uptake determination. Between days 7 and 14 of the experiment, tracer uptake by low concentration larvae increased nearly threefold. During this same period uptake by control larvae also increased by a factor of two (see Discussion). A one-tailed Mann-Whitney test revealed that cumulative radio-tracer uptake by low concentration larvae was significantly greater than for control larvae.

Larval radioactive tracer uptake was measured for all remaining replicates upon termination of the experiment on day 18. Between day 14 and termination of the experiment, tracer uptake by low concentration larvae increased markedly, whereas uptake by control larvae increased only slightly. A one-tailed Mann-Whitney test indicated that tracer uptake by low concentration larvae was significantly elevated when compared with control larvae.

3) Survivorship. Larval survivorship was low in the control and all WSF-test solution exposure regimes over the 18-day exposure period; however, there was a direct relationship between larval mortality and exposure concentration (Figure 11-16).

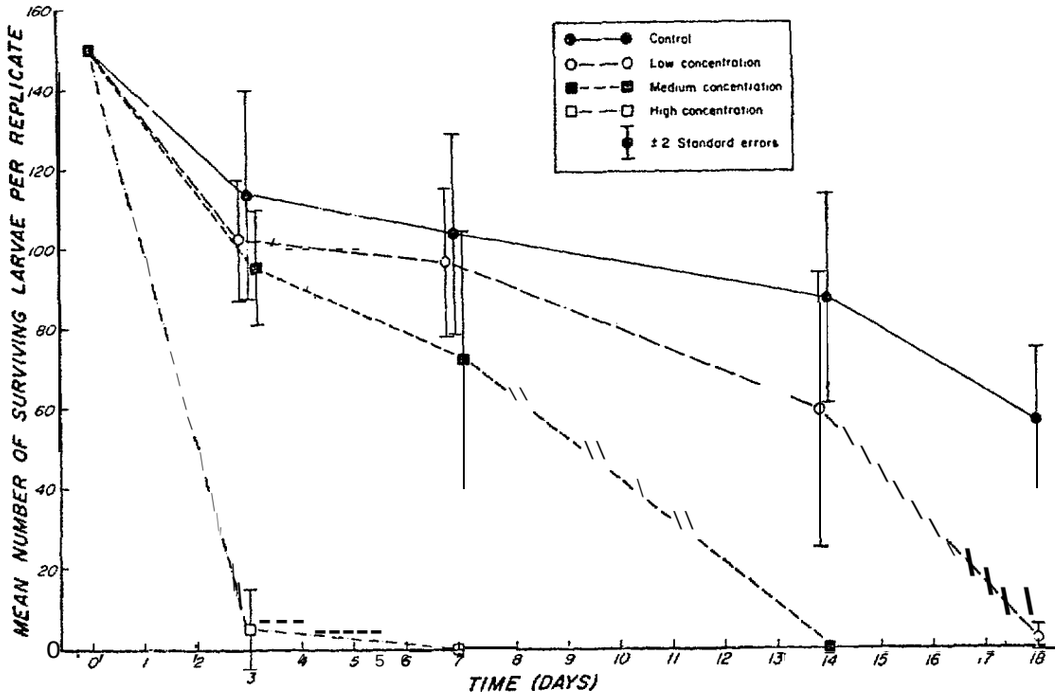

Figure 11.16, Mean number of surviving larval California halibut following 3, 7, 14, and **18** days of **test solution** exposure.

Larval survivors hip was determined for three replicates from the control and each WSF exposure regime following three days of exposure. The same initial number of larvae was placed in each replicate, thus, the mean number of surviving larvae was determined from direct counts in each replicate. The mean number of surviving larvae/replicate ranged from 5.0 larvae in the high concentrationto 113.67 larvae in the control (Table II-27 and data appendix) and was inversely proportional to the exposure concentration. A K-W ANOVAbased on ranked data, however, indicated there was no significant difference between larval survivorship in the control and test solution exposure regimes.

Three replicates were sacrificed from the control and each test solution exposure regime on day 7 of the experiment. The mean number of surviving larvae had declined only slightly in the control, low, and medium concentrations compared with day 3. In contrast, larval mortality

Table II-27 . Mean number of surviving Paralichthys californicus larvae after 4, 7, 14 and 18 days of test solution exposure. Standard errors are shown in parentheses.

Concentration	Exposure Period (Days) 0	4[a]	7[a]	14[b]	18[c]
Control	150	113.67 (15.17)	103.33 (14.54)	87.00 (15.17)	56.33 (10.35)
Low	150	102.33 (8.69)	96.33 (10.86)	59.00 (19.85)	?.33 (1.54)
Medium	I 50	95.33 (8.40)	72.00 (18.56)	---	---
High	150	5.00 (5.78)	---	---	---

[a] Number of surviving larvae not significantly different (p>0.05) among groups; k-WP.NOVA.

[b] Number of surviving larvae not significantly different (P >0.05) among groups; Mann-Whitney test.

[c] Number of surviving larvae significantly different (p <0.05) among groups; Mann-Whitney test.

in the high concentration exposure regime was 100.0% (Table II-27 and Figure 11-16). Mean numbers of surviving larvae ranged between 72,0 and 103.3 individuals/replicate in the medium concentration and control, respectively, and were inversely proportional to exposure concentration. A K-W ANOVA failed to demonstrate a significant difference in the number of larvae surviving between the control, low, and medium concentration exposure regimes.

Following 14 days of exposure three replicates were terminated from the control and low concentration exposure regimes, while the six remaining replicates were terminated from the medium concentration exposure regime. No live larvae were found in any of the medium concentration replicates. The mean number of surviving larvae ranged between 59.0 and 87.0 larvae/replicate in the low concentration and control, respectively, (Table 1',-27 and Figure 11-16). A one-tailed Mann-Whitney test failed to demonstrate a significant difference in larval survivorship between the control and low concentration exposure regimes.

The larval experiment was terminated after 18 days by sacrificing all remaining replicates from the control and low concentration regimes. Between days 14 and 18 of the experiment, the numbers of surviving larvae markedly declined in the low concentration (2.33), but only slightly in the control. A one-tailed Mann-Whitney test demonstrated that larval mortality was significantly greater in the low concentration than in the control.

4) Growth. Mean total length (mm) of developing larvae was used as a measure of larval growth. Total length (TL) measurements were recorded for up to 20 larvae in each replicate sacrificed from the control and test solution exposure regimes on days 3, 7, 14 , and 18 of the experiment. The size of larvae at hatching was assumed to be 2.29 mm TL in each replicate based on measurements of newly-hatched larvae from a parallel static experiment.

Table II-28. Mean total length of larval P. californicus (mm) surviving after 3, 7, 14, and 18 days of test solution exposure. Standard errors are shown in parentheses.

Concentration	Exposure Period (Days) 0	3[a]	7[a]	14[b]	18[b]
Control	2.29 (0.05)	2.60[c] (0.09)	3.09[c] (0.03)	4.23 (0.07)	4,60 (0.12)
Low	2.29 (0.05)	2.74[c] (0.03)	3.06[c] (0.02)	3.80 [0.08)	4.02 [0.32)
Medium	2.29 [0.05)	2.71[c] (0.02)	2.71[d] (0.03)	--	--
High	2.29 (0.05)	2.07[d] (0.07)	--	--	--

a Total length of larvae significantly different ($p < 0.05$) among groups; K-W ANOVA.

b Total length of larvae significantly different ($p < 0.05$) among groups; Mann-Whitney test.

c,d Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

In general, larval growth in the control and low concentration regimes followed a similar pattern of increase over the 18-day experiment (Table II-28; Figure 11-17; data appendix]. No significant difference in growth was evident after seven days of exposure, although some divergence *in growth* rates occurred between 7 and 18 days, with low concentration larvae exhibiting a lower growth rate. After 14 days of exposure and continuing through termination of the experiment (day 18), larval growth was significantly depressed in the low concentration regime when compared with the control (Table 11-28).

Medium concentration larvae exhibited growth comparable to larvae in the control and low concentration regimes over the first three days of exposure (Figure 11-17). Between days 3 and 7, however, little change in size was observed. After seven days, larval growth in the medium concentration

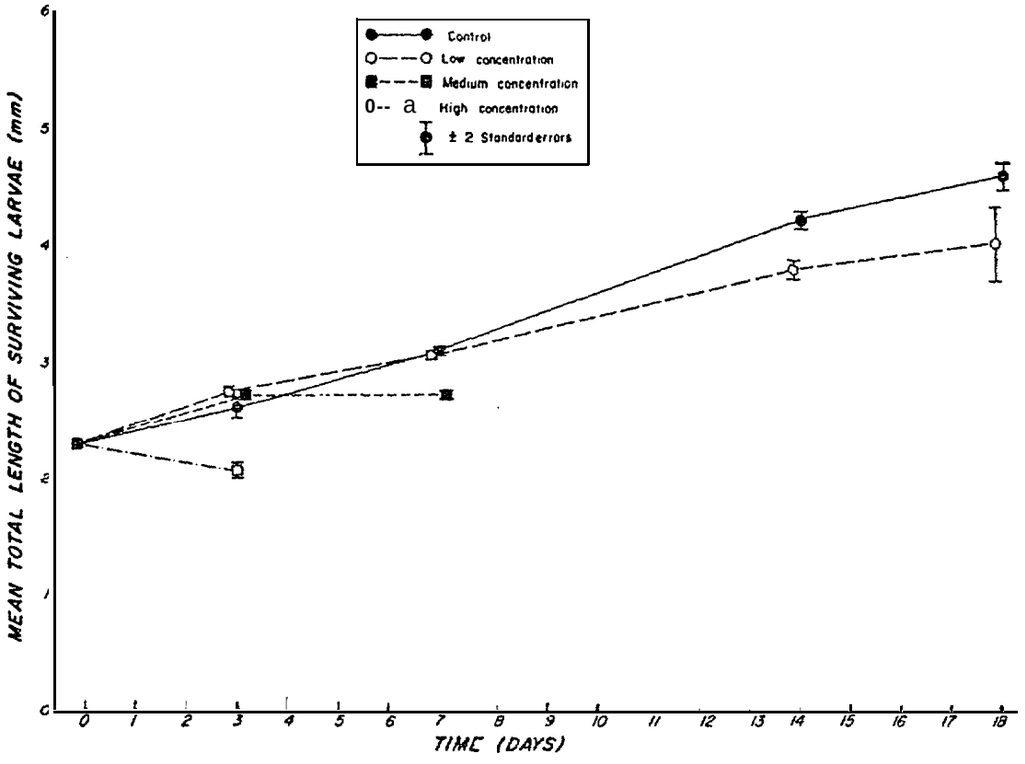

Figure **U-17. Mean total length cd surviving larval California halibut following 3,7, 14, and 18 days of test solution exposure.**

regime was significantly depressed compared with the control and low concentration (Table 11-28). Medium concentration larvae suffered 100% mortality between days 7 and 14; thus no subsequent estimates of growth were obtained.

Because of extremely high mortality in 'he high concentration exposure regime, larval growth could only be measured after three days of exposure. At that time, growth of high concentration larvae was significantly depressed compared with larvae in the control and all other test solution exposure regimes.

5} Development. Development of larval California halibut was assessed after 3, 7, 14 and 18 days of test solution exposure. Several morphological characters including snout-vent (S-V) length, yolk sac diameter, eye diameter, otic vesicle diameter, and eye to-tip-of-snout (ETS) distance were measured on larvae from each exposure regime.

Following three days of exposure, larvae in the high concentration were generally less well developed than larvae from the control, low or medium concentration ex. osure regimes (Plate II-2a-d). In particular, the head and posterior gut (i.e. vent) regions were less well developed and smaller, while the eye and otic vesicle diameters were significantly reduced in size (Plate II-2a-d; Table 11-29). Although high concentration larvae were significantly shorter in total length (Table II-28 and Figure 11-17), there was no significant difference in S-V length among control and WSF-exposed larvae (Table

Plate II-2(a-d). Representative photographs of whole mounted larval P. californicus from the control group (a) and low (b), medium (c) and high (d) concentration exposure regimes following three days of exposure. Structures labeled as follows: eye {E), otic vesicle (0), vent (V), and yolk (Y). See text Tables 11-28 through II-30 and Figure 11-17 for significance of morphometrics. 43X.

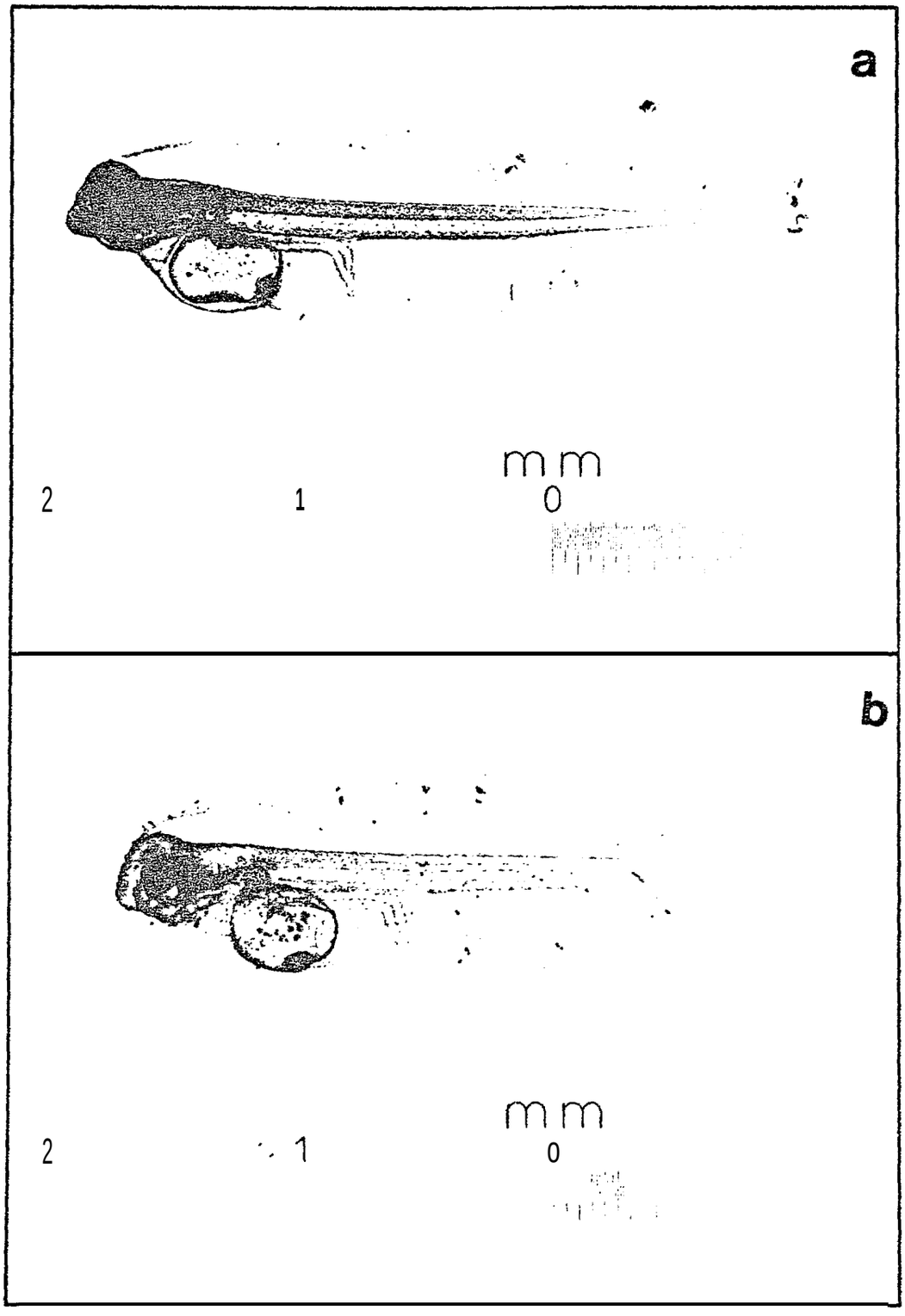
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Plate II-2(a-d). (Cont)

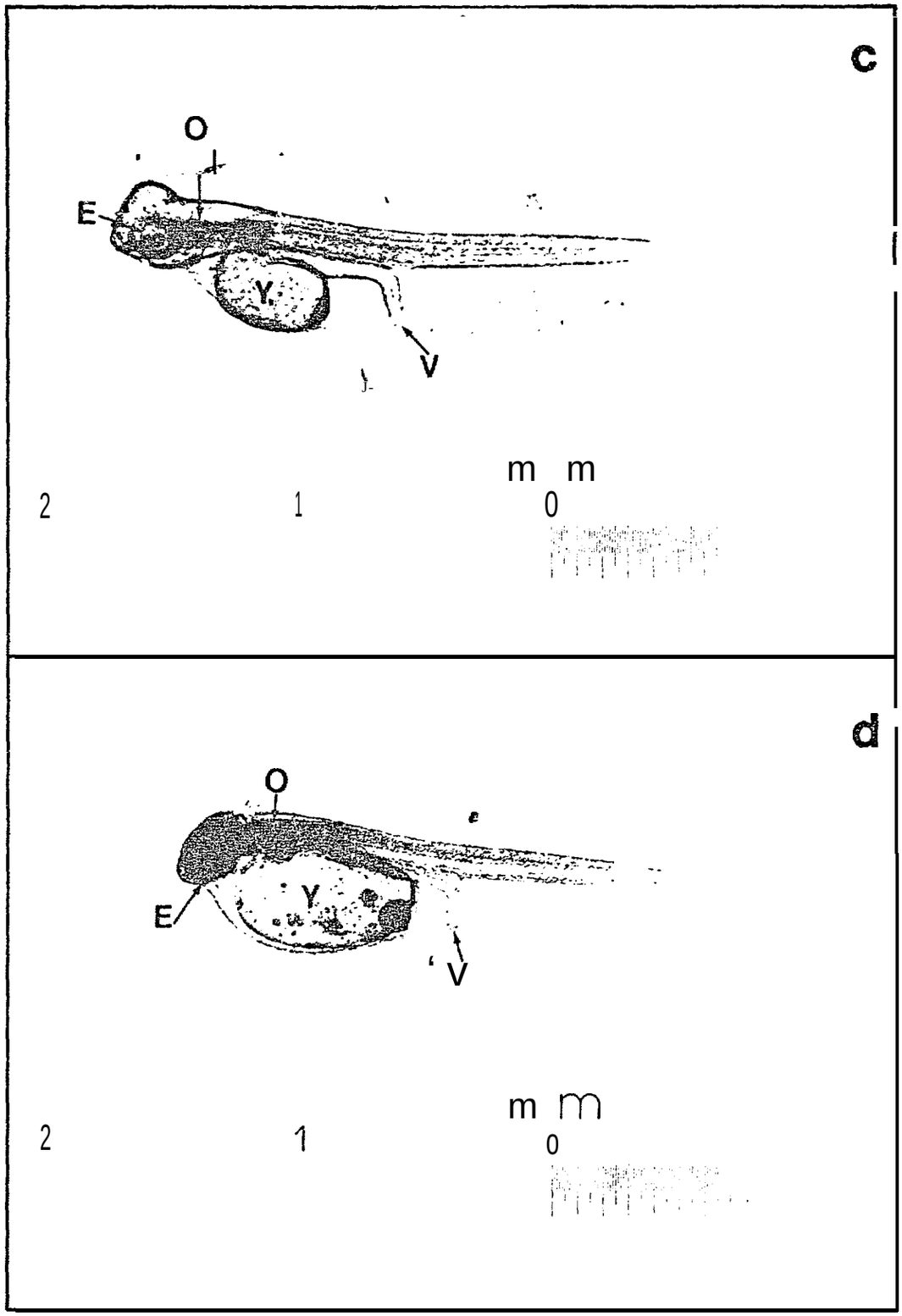
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Table II-29. Mean snout-vent length, yolk-sac diameter, eye diameter, otic vesicle diameter, **and distance from** eye **to tip of snout for surviving** *P. calif ornicus* larvae after **3 and 7** days of **test** solution exposure. **Standard** errors are shown **in** parentheses. **Each** mean based on the measurement of six larvae.

Morphometric Measurement (mm)	Control	Low	Medium	High
	3 Day Exposure			
Snout-vent length[c]	1.31 (0.01)	1.29 (0.01)	1.30 (0.01)	1.29 (0.01)
Yolk-sac: Sum of anterior-posterior and dorsal-ventral diameters[d]	0.81[e] (0.02)	0.80[e] (0.02)	0.79[e] (0.02)	1.17[f] (0.03)
Diameter of Eye: Anterior-posterior[d]	0.21[e] (0.012)	0.22[e] (0.012)	0.20[e] (0.004)	0.14[f] (0.004)
Diameter of Otic Vesicle: Anterior-posterior[d]	0.12[e] (0.004)	0.12[e] (0.004)	0.12[e] (0.004)	0.05[f] (0.004)
Distance from Eye to Tip of Snout[c]	0.14 (0.004)	0.14 (0.004)	0.14 (0.004)	0.12 (0.004)
	7 Day Exposure			
Snout-vent length[c]	1.38 (0.01)	1.35 (0.02)	1.37 (0.008)	a
Yolk-sac: Sum of anterior-posterior and dorsal-ventral diameters[b]				
Diameter of Eye: Anterior-posterior[c]	0.21 (0.012)	0.22 (0.008)	[illegible]	
Diameter of Otic Vesicle: Anterior-posterior[d]	0.18[e] [illegible]	0.17[e] (0.008)	0.12[f] (0.004)	
Distance from Eye to Tip of Snout[d]	0.14[e] (0.004)	0.14[e] (0.008)	0.12[f] (0.008)	

a All high concentration larvae dead prior to day 7 of experiment.

b Yolk-sac fully absorbed by day 7 of experiment.

c No significant difference ($p > 0.05$) among groups; K-ii ANOVA.

d Significant difference ($p < 0.05$) among groups; K-W ANOVA.

e,f Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric analog to SNK multiple comparisons test. Groups with same letter not significantly different.

11-29). In contrast, the yolk-sac diameter of high concentration larvae was significantly larger than for larval control, low or medium concentrations (Plate 11-2a-d; Table 11-29).

Although medium concentration larvae were significantly reduced in total length after 7 days of exposure (Table II-28 and Figure 11-17), there was no significant difference in S-V length between control and WSF-exposed larvae (Table II-29). However, there was clear evidence that the anterior head region and jaws of medium concentration larvae were markedly less well developed when compared with control and low concentration larvae (Plate II-3(a-c); Table ii-29). Furthermore, the proportion of medium concentration larvae with poorly developed jaws was significantly greater than in the control or low concentration exposure regime (Table 11-30).

Following 14 days of exposure and upon termination of the experiment on ddy 18, larval development was. assessed in the control and low concentration regimes. There was no evidence of delayed, reduced, or abnormal development in the low concentration at either day 14 or 18 (Plates n-4a-b and 11-5a-b), and no significant difference in either the S-V length, eye diameter or ETS distance (Table 11-31).

6) Histopathology. As discussed previously, histological examination of target organs in larvae was not feasible. However, whole organism mounts were prepared and subjected to microscopic examination (See Section C.2.b.5), Development).

7) Behavior. Larvae in the control and WSF test solution concentrations were spread throughout the water column after hatching. Short bursts of larval movement were observed in all exposure regimes on day 2 although most of their time was passively spent floating in the water column. No larvae were visible in any high concentration replicates OR day 3, whereas larvae in all other exposure regimes were spread throughout the water column and occasionally exhibited short bursts of forward movement. Examination of high concentration replicates on day 3 revealed that only a few larvae remained alive. These larvae exhibited little movement and quickly sank to the bottom of the container when not disturbed.

Table II-30. Number of normal and malformed larval California halibut after 7 days 01 exposure. Proportions of larvae in each category are shown in parentheses.

Concentration	Number of Normal Larvae[a]	Number of Malformed Larvae
Control	303 (0.97)[c]	7 (0.03)[b]
Low	282 (0.97)[c]	7 (0.03)[b]
Medium	0 (0.0)[b]	216 (1.0)[c]

a Proportion of larvae with development abnormalities significantly different ($p < 0.05$) among groups; Chi-square analysis.

b,c, d Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

Table II-31. Mean snout-vent length, yolk-sac diameter, eye diameter, otic vesicle diameter, and distance from eye to tip of snout for surviving P. californicus larvae after 14 and 18 days of test solution exposure. Standard errors shown in parentheses. Each mean based on the measurement of six larvae.

Morphometric Measurement (mm)	14 Day Exposure[a] Control	Low
Snout-vent Length[b]	2.0 (0.04)	1.93 (0.03)
Diameter of Eye: Anterior-posterior[b]	0.29 (0.012)	0.29 (0.008)
Distance from Eye to Tip of Snout[b]	0.17 (0.004)	0.17 (0.004)
	18 Day Exposure[a]	
Snout-vent Length[b]	2.26 (0.04)	2.16 (0.03)
Diameter of Eye: Anterior-posterior[b]	0.30 (0.04)	0.32 (0.02)
Distance from Eye to Tip of Snout[b]	0.19 (0.008)	0.19 (0.008)

a All medium and high concentration larvae died prior to day 14 of the experiment.

b No significant difference ($p > 0.05$); K-U ANOVA.

Food was added to the control, low and medium concentration regimes on day 4 of the experiment. A behavior sequence consisting of several short forward bursts of movement followed by a passive floating period was observed in all exposure regimes. Larvae in the medium concentration began to exhibit reduced movement by day 5, whereas larvae in the control and low concentrations appeared to be actively seeking food. Larvae in the medium concentration regime generally floated head down and only occasionally exhibited movement. Larvae in the control and low concentration exposure regimes were actively moving on day 6. Behavior of these larvae consisted of several short swimming bursts or "strikes", followed by continued swimming or a period of passive floating. Larvae were generally oriented in a horizontal position when floating. The general behavior of larvae in the medium concentration regime was characterized by a head down position in the water column, with occasional bursts of forward movement.

No change in larval behavior was noted through days 7 and 8 of the experiment. Examination of larvae from the medium concentration on day 8 indicated they would swim continuously only when disturbed. Whenever larvae were left undisturbed, their general behavior consisted of floating head down with only occasional attempts at forward movement. The forward movement of these larvae appeared to be an attempt to maintain position in the water column rather than to feed. Examination of larval gut contents on day 8 revealed that over 90% of the larvae from the control and low concentration exposure contained food. while only 11% of the larvae from the medium concentration exposure contained food (Table II-32).

Table II-32. Larval California halibut examination of gut for food content.

Concentration	Mean Number of Larvae	Mean Number of Larvae with Food in Gut	Mean % Larvae With Food in Gut
Control	103.3	100.3	97.3
Low	96.3	88.0	90.7
Medium	72.0	8.0	11.2

Between days 9 and 11 of the experiment, larval behavior did not noticeably change. Larvae in the control and low concentration

Plate II-3(a-c). Representative photographs of whole mounted larval *P. californicus* from the control group (a) and low (b) and medium (c) concentration exposure regimes following 7 days of exposure. Structures labeled as follows: gut (G), otic vesicle (0), mandible (M), snout (S), and vent (V). 43X.

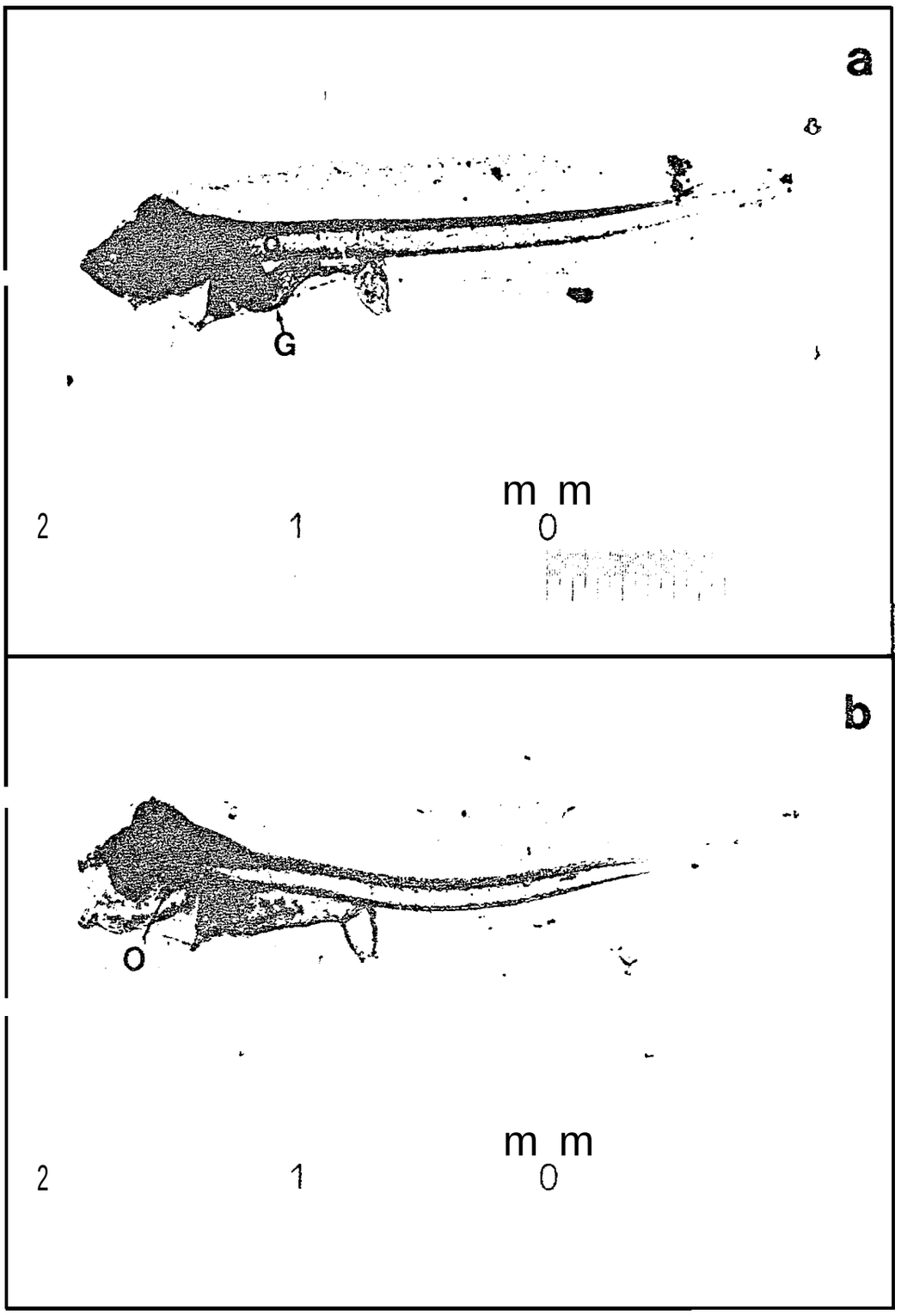

a
G
m m
2
1
0
b
O
m m
2
1
0

Plate II-3(a-c). (Cent)

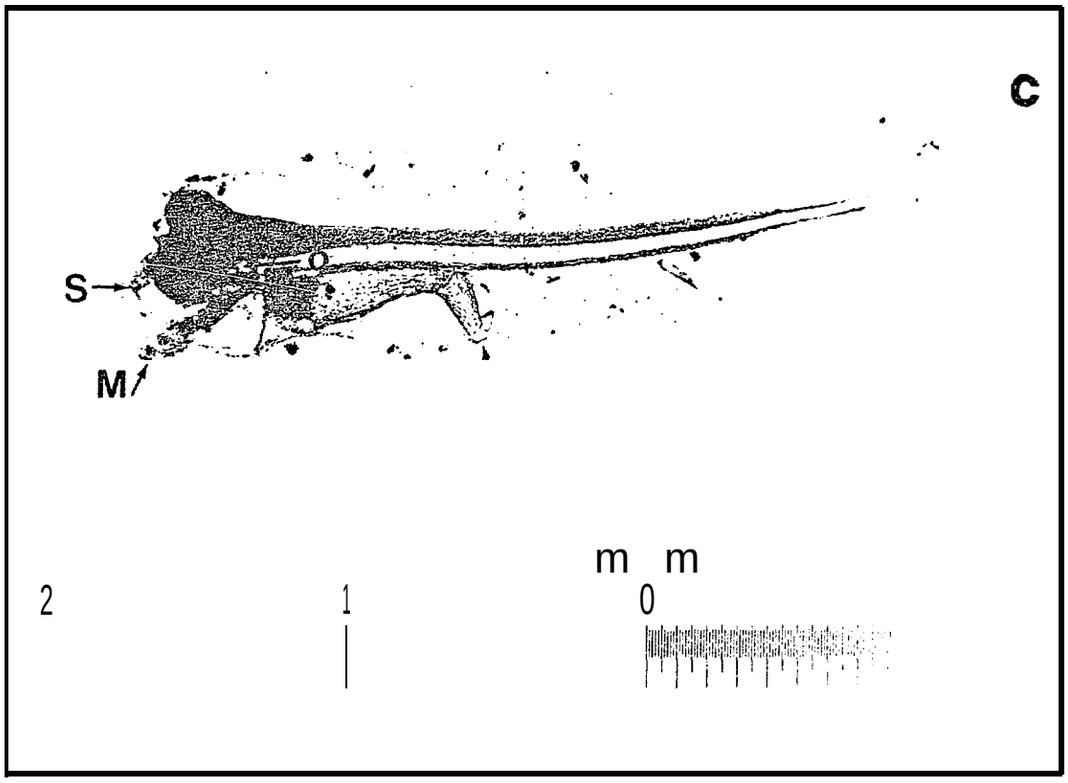

c
S
M
m m
2
1
0

Plate 11-4(a-b). Representative photographs of whole mounted larval *P. californicus* from the control group (a) and low (b) concentration exposure regime after 14 days of exposure. Structures labeled as follows: heart (H), liver (L). 43X.

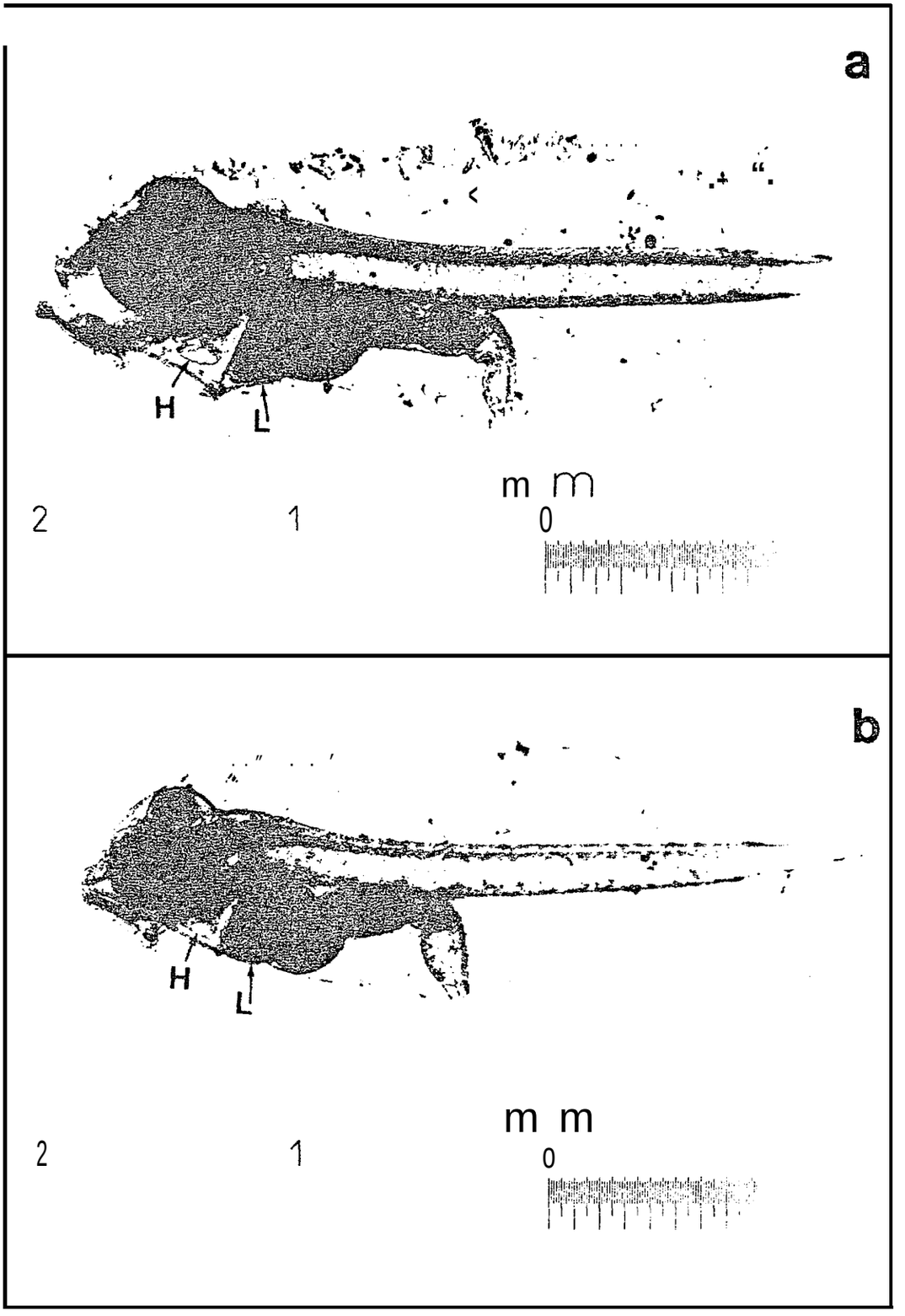
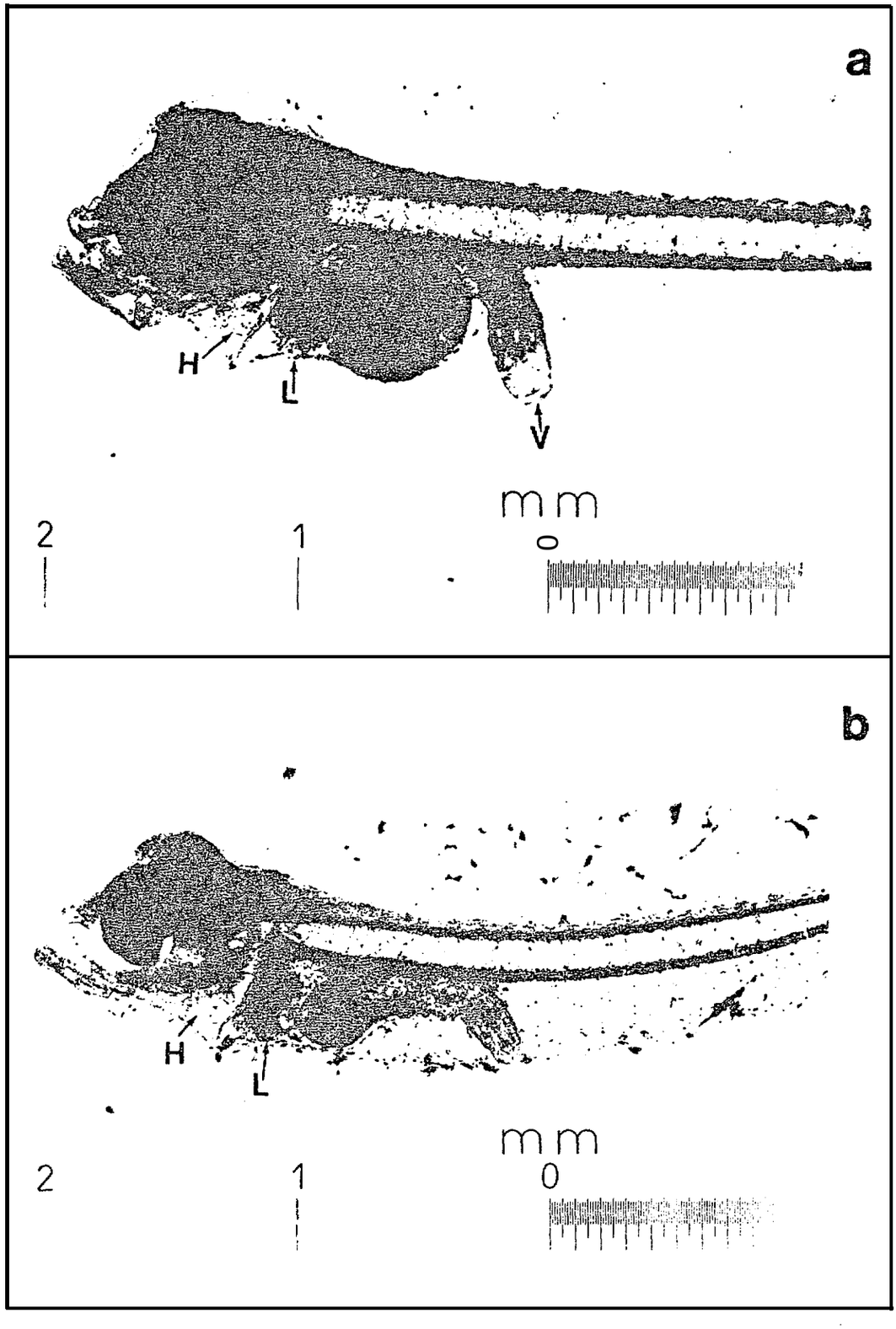

a
H
L
m m
2
1
0
b
H
L
m m
2
1
0

Plate II-5(a-b). Representative photographs of whole-mounted larval P. californicus from the control group (a) and low (b) concentration exposure regimes following 18 days of exposure. Structures labeled as follows: heart (H), liver (L) and vent (V). 43X.


a
H
L
V
mm
2
1
0
b
H
L
mm
2
1
0

exposure regimes were spread throughout the water column and actively moved about the test aquaria. Their movement was similar to that observed earlier in the experiment. No change was observed in the behavior of larvae from the medium concentration regime, although no larvae were visible in the medium concentration exposure regime after day 11. By day 12, larval behavior in the control and low concentration exposure regimes consisted primarily of swimming with occasional "striking" motions. Between days 14 and 17, larvae in both the control and low concentration regimes remained very active and moved freely throughout the water column.

c. Adult Experiment

1) Hydrocarbon Exposure. Hydrocarbon exposure data for the 80-day adult halibut (Paralichthys californicus) experiment are presented in Table II-33. Control and low exposure level hydrocarbon concentrations were Statistically different at the 95% significance level (Student's t-test). When the mean background hydrocarbon level was subtracted from the mean test solution concentrations, the resulting hydrocarbon concentrations were very close to the target values for all three dosing levels. The values obtained after background hydrocarbon correction were 4.3, 41.4 and 412.0 µg/l, respectively. Furthermore, all three test solution exposure regimes exhibited a consistent hydrocarbon composition over the entire 80-day study and differed from the control level of 5.2 µg/l.

Table H-33. Hydrocarbon exposure concentrations for California halibut adult experiment.

Exposure Level	Hydrocarbon Concentration (µg/l)[a] Volatile Fraction	Extractable Fraction[b]	Total[c]
Cent rol	0.5: 0.3	4.7+1.8 [13.0+4.0]	5.2+ 1.9 [13.5+ 4.0]
Low	5.2+ 9.5	4.3+1.8 [11.63.81	9.5+ 5.0 [16.8+ 5.6]
Medium	37.0<23.0	9.6+5.1 [17.6+6.3]	46.6+24.1 [54.6+23.4]
High	244+101	173+106 [183+102]	417+145 [427+143]

a where appropriate, data are reported as the mean ±1 standard deviation.

b Extractable hydrocarbon values are reported corrected for the presence of diethyl tetrahydrofuran and uncorrected values are also presented in brackets.

c Total hydrocarbon values represent the sum of the average daily volatile fraction plus the weekly extractable fraction. Values are also reported both corrected and uncorrected for diethyl tetrahydrofuran.

2) Hydrocarbon Tissue Burdens. Fish were sacrificed and selected tissues were analyzed for petrogenic hydrocarbon tissue burdens upon termination of the adult halibut experiment. Tissues selected for detailed characterization included the eye, gonad, digestive tract, liver, gill, and muscle. Each of these tissues was extracted and analyzed for parent petrogenic hydrocarbon content. With the exception of muscle tissue, all samples were composites from several fish. Halibut muscle samples from both individual organisms and composite tissues were analyzed for comparison and evaluation of individual variability.

Petrogenic hydrocarbon tissue burden data are presented as a series of tabular summaries. Hydrocarbon data are grouped into four general categories for presentation. These categories are composed of the parent compound and its alkyl-substituted homologs including: benzenes, naphthalenes, thiophenes and furans, and phenanthrenes. Additionally, each table contains a fifth category, total petrogenic hydrocarbons identified, which represents the summation of the four categories described above. Completetissueburden characterizations which detail individual compounds and corresponding concentration data are contained in the data appendix.

Tables II-34 and II-35 present results of tissue burden characterizations for the eye and combined gonadal (testis and ovary) tissues, respectively. Relatively low levels of petrogenic hydrocarbons were identified

Table II-34. Petrogenic hydrocarbon burden in adult California halibut eye tissue.

Toxicant Category[b]	Bkg[c]	Exposure Levels: Cent rol	Low	Medium	High
Benzenes	ND	ND	ND	ND	ND
Naphthalenes	ND	ND	ND	*	14(J
Thiophenes and Furans	ND	ND	ND	ND	ND
Phenanthrenes	ND	ND	ND	ND	ND
Total Petrogenic Hydrocarbons Identi fi ed	ND	ND	ND	•	140

NO = None detected. * = Trace amount detected.

a Concentration data reported as mean ±1 standard deviation in ng/g dry weight.

b Each category is composed of the parent compound and alkyl-substituted homologs.

c Bkg = background tissue sample [tissue sample from Organisms collected at the same time as those used for exposure studies).

Table II-35. Petrogenic hydrocarbon burden in adult California halibut gonad tissue.

Toxicant Category[b]	Bkg[c]	Exposure Levels: Cant rol	Low	Medium	High
Benzenes	ND	ND	*	*	87
Naphthalenes	NO	ND	ND	•	430
Thiophenes and Furans	NO	ND	ND	*	*
Phenanthrenes	ND	NO	ND	ND	ND
Total Petrogenic Hydrocarbons Identified	ND	ND	*	*	517

ND = None detected. · · Trace amount detected.

a Concentration data reported as mean ±1 standard deviation in ng/g dry weight.

b Each category is composed of the parent compound and alkyl-substituted homologs.

c Bkg = Background tissue sample (tissue sample from organisms collected at the same time as those used for exposure studies).

in both tissues. However, in both cases available tissue masses were limited, and as a result analytical sensitivities were reduced relative to other selected tissues. Sensitivities were reduced by a factor of 4 to 6 for eye extracts and by a factor of 3 to 14 for gonad extracts. The reduced detection limits complicate interpretation of these data relative to other selected tissues.

No petroleum derived hydrocarbons were identified in eye extracts from background, control and low concentration fish and only a trace was detected in tissues from fish in the medium concentration. Extracts from fish exposed to the highest concentrations were found to contain 140 rig/g of naphthalene derivatives. Gonad tissue extract results closely paralleled eye extracts. No petrogenic hydrocarbons were detected in tissues from either background or control fish. Low and medium concentration tissue samples showed traces of several petrogenic hydrocarbon groups including benzenes, naphthalenes and thiophenes. Gonad tissues from fish exposed to the high concentration contained a petrogenic hydrocarbon content of 517 rig/g, which was largely composed of naphthalene derivatives.

Tables II-36, II-37, and II-38 present bioaccumulation data for petrogenic hydrocarbons in muscle tissue from experimental P. californicus. Data obtained from analysis of composite tissue samples, Table II-36, indicate that no detectable levels of petroleum hydrocarbons were identified in background, control and medium concentration tissue extracts. However, low levels of a C_3-substituted benzene were identified in muscle extracts from fish exposed to the low concentration. Tissue extracts from fish exposed to the high concentration were found to contain significant amounts of both benzene and naphthalene derivatives at a mean combined concentration of 436 rig/g.

Triplicate hydrocarbon characterizations were also performed on muscle tissue from individual organisms at each exposure regime. Results of these analyses are presented in summary format as Table II-37. As evidenced by this table, bioaccumulation data on muscle tissue from individual fish were very similar to those observed for composite samples. No detectable levels of petrogenic hydrocarbons were observed in control fish, and only trace amounts were detected in fish from the low and medium exposure levels. A mean tissue burden of 323 rig/g was observed in fish from the high concentration of which 231 rig/g was attributable to naphthalene and its alkyl-

Table II-36. Petrogenic hydrocarbon burden in adult California halibut muscle tissue composite from random subsample within an exposure level.

Toxicant Category[b]	Bkg[c]	Exposure Level[a] Control	Low	Medium	High
Benzenes	ND	ND	14+18	ND	196+34
Naphthalenes	ND	ND	ND	ND	240+21
Thiophenes and Furans	ND	ND	ND	ND	NO
Phenanthrenes	ND	NO	ND	ND	NO
Total Petrogenic Hydrocarbons Identified	ND	ND	14+18	ND	436+55

ND = None detected.

[a] Concentration data reported as mean +1 standard deviation in ng/g dry weight.

b Each category is composed of the parent compound and alkyl-substituted homologs.

[c] Bkg = Background tissue sample (tissue sample from organisms collected at the same time as those used for exposure studies) .

Table II-37. Petrogenic hydrocarbon burden in adult California halibut muscle. Data pooled from three individuals/exposure level (see Table II-38).

Toxicant Category[b]	Bkg[c]	Exposure Levels Control	Low	Medium	High
Benzenes	t	ND	ND	NO	75+37
Naphthalenes	t	N	D *	*	231+9
Thiophenes and Furans	t	ND	ND	ND	16+3
Phenanthrenes	t	ND	NO	ND	ND
Total Petrogenic hydrocarbons Identified	t	ND	*	*	323+41

ND = None detected. * = Trace amount detected.
t Sample type not analyzed.

a Concentration data reported as mean +1 standard deviation in rig/g dry weight.

b Each category is composed of the parent compound and alkyl -substituted homologs.

c Bkg = Background tissue sample (tissue sample from organisms collected at the same time as those used for exposure studies].

substituted homologs. Although mean hydrocarbon tissue burdens from high concentration individual and composite muscle samples were statistically different at the 95% significance level (Student's test), overall the data compare favorably given the inherent limitations of the analytical protocol employed. Complete data for each replicate from individual fish are presented in l-able II-38.

Bioaccumulation data for digestive tract and liver target tissues are summarized in Tables II-39 and 11-40, respectively. The bioaccumulation pattern observed was very similar for both tissues. Petrogenic hydrocarbons were not detected in the background, control, low and medium concentration samples. However in both cases, significant levels of petrogenic hydrocarbons were identified at the high concentration. As Table II-39 indicates, a mean total tissue burden of 1170 rig/g was found to accumulate in the digestive tract at the high concentration. The hydrocarbons identified included a full spectrum of alkyl-substituted benzenes and naphthalenes, as well as several substituted thiophenes.

Ta **Table II-38. Petrogenic hydrocarbon burden in adult California halibut muscle tissue from individual organisms.**

	Exposure Level a Control			Low			Medium			High		
Fish 1.0.	008	107	025	065	018	050	045	087	096	094	093	062
Tank I.D.	0-2	D-3	O-3	c-1	c-2	c-3	B-1	B-2	8-3	A-1	A-2	A-3
Benzenes[b]	ND	ND	NO	ND	ND	ND	NO	ND	ND	68	43	115
Naphthalenes[b]	ND	NO	ND	*	*	*	*	*	*	223	231	240
Thiophenes and Furans[b]	ND	ND	NO	NO	ND	NO	NO	NO	ND	19	16	14
Phenanthrenes[b]	ND	NO	NO	NO	ND	ND	NO	NO	ND	NO	ND	ND
Total Petrogenic Identified Hydrocarbons	NO	ND	NO	*	*	*	*	*	*	310	290	369

ND = None detected. * = Trace amount detected.

a Concentration data reported as ng/g dry weight.

b Each category is composed of the parent compound and alkyl-substituted homologs.

Table II-39. Petrogenic hydrocarbon burden in adult California halibut digestive tract tissue.

Toxicant Category[b]	Bkg[c]	Exposure Level[a] Control	Low	Medium	High
Benzenes	ND	ND	ND	ND	627L339
Naphthalenes	ND	ND	ND	ND	512±99
Thiophenes and Furans	NO	ND	ND	ND	32±1 2
Phenanthrenes	ND	ND	ND	ND	ND
Total Petrogenic Hydrocarbons Identified	ND	ND	ND	MU	1170<282

ND = None detected.

[a] Concentration data reported as mean ±1 standard deviation in ng/g dry weight.

b Each category is composed of the Parent compound and alkyl-substituted homologs.

c Bkg = Background tissue sample [tissue sample from organisms collected at the sane time as those used for exposure studies].

Table II-40. Petrogenic hydrocarbon burden in adult California halibut liver tissue.

Toxicant Category[b]	Bkg[c]	Exposure Levels Control	Low	Medium	High
Benzenes	ND	HO	ND	ND	2118±124
Naphthalenes	ND	ND	ND	ND	3257±773
Thiophenes and Furans	ND	NO	ND	ND	ND
Phenanthrenes	ND	ND	ND	ND	ND
Total Petrogenic Hydrocarbons Identified	ND	ND	ND	ND	5374+649

ND = None detected.

a Concentration data reported as mean ±1 standard deviation in rig/g dry weight.

b Each category is composed of the parent compound and alkyl-substituted homologs.

c Bkg = Background tissue sample (tissue sample from organisms collected at the same time as those used for exposure studies).

Liver tissue from adult halibut exposed to the high concentration regime showed a mean petrogenic hydrocarbon concentration of over 5300 rig/g. Of this total, approximately 2100 rig/g was attributable to benzene derivatives and 3200 rig/g to naphthalene constituents. Individual alkyl benzenes ranged from 120 to 930 rig/g and naphthalenes ranged from 530 to 950 rig/g. Eight individual alkyl benzenes and four alkyl naphthalenes were identified. Problems associated with preparing liver samples for analysis may have resulted in higher detection limits for some hydrocarbon species, thus reported values should be considered conservative estimates.

A summary of petrogenic hydrocarbon tissue burden data from adult halibut gill samples is presented in Table 11-41. Gill samples from all three exposure levels showed the highest tissue burdens of the six target tissues examined. No petrogenic hydrocarbons were detected in background and control samples; however, significant levels were found in tissue extracts from exposed fish. Mean petrogenic hydrocarbon concentrations of 63, 145, and 6916 rig/g were detected in gill tissues from organisms exposed to the low, medium and high concentration test solutions, respectively. A single C_4-substituted benzene was identified in low concentration tissue extracts while medium and high concentration extracts showed alkyl-substituted derivatives of benzene, naphthalene and benzothiophenes. Halibut gill extracts exhibited the greatest diversity of individual hydrocarbons, with a total of 28 individual aromatics identified in tissue extracts from animals exposed to the high concentration regime.

A summary of petrogenic hydrocarbon tissue burden data for each of the six selected tissues from the adult California halibut study is given in Table II-42. These data are presented as total tissue burdens, representing the summation of all compounds which could be directly linked to the parental crude oil toxicant and distinguished from hydrocarbons of biogenic origin.

In addition to characterizing tissue burdens for petrogenic hydrocarbons, it is of interest to make quantitative determination of bioaccumulation factors. Bioaccumulation factors are a ratio of the aqueous exposure level relative to the resultant tissue burden. As such, bioaccumulation factors are quantitative measurements of toxicant uptake over the course of the exposure period, and provide a means of correlating the accumulation of one chemical toxicant relative to others.

Table II-41. Petrogenic hydrocarbon burden in adult California halibut gill tissue.

Toxicant Category[b]	Bkg[c]	Exposure Levels Control	Low	Medium	High
Benzenes	ND	ND	63±57	85±76	2890±2694
Naphthalenes	ND	NO	ND	52±72	3716±1759
Thiophenes and Furans	ND	ND	ND	8±12	294±359
Phenanthrenes	ND	ND	ND	ND	*
Total Petrogenic Hydrocarbons Identified	ND	ND	63±57	145±135	6916~4751

ND = None detected. * = Trace amount detected.

[a] Concentration data reported as mean ±1 standard deviation in rig/g dry weight.

b Each category is composed of the parent compound and alkyl-substituted homologs.

c Bkg = Background tissue sample [tissue sample from organisms collected at the same time as those used for exposure studies).

Table II-42. Summary of petrogenic hydrocarbon burden in adult California halibut tissue.

Tissue	Bkgb	Exposure Level[a] Control	Low	Medium	High
Gonad	N D		N D	* *	517
Eye	ND	ND	ND	•	140
Muscle (Composite]	ND	ND	14±18	ND	436±55
Muscle (individual Fish)	†	ND	*	*	323±41
Liver	ND	NO	ND	ND	5374±649
Gill	ND	ND	63±57	145±135	6916±4757
Digestive Tract	ND	ND	ND	ND	11 70+282

NO = None detected * = Trace amount detected
t = Sample type not analyzed

a Where replicate samples were analyzed, concentration data are reported as mean ±1 standard deviation in rig/g dry weight. Values reported are total identifiable petrogenic hydrocarbon tissue burdens.

b Bkg . Background tissuesample(tissuesample from organisms collected at the same time as those used for exposure studies).

With few exceptions it was difficult to calculate bioaccumulation factors in the current toxicity studies because of the low concentrations of individual hydrocarbons in the aqueous exposure solutions. In many cases, hydrocarbons which were identified in the parent crude toxicant, and subsequently identified in tissue extracts, were not present at detectable levels in the aqueous test solutions. In such cases, tissue levels observed in exposed organisms can only be roughly related to known detection limit values for these compounds to develop conservative bioaccumulation factor approximations. For example, if the aqueous detection limit for a given hydrocarbon is known to be 0.1 µg/l and the tissue extract shows a level of 100 rig/g, a conservative estimate of the bioaccumulation factor for this compound would be 1000. The assumption implicit in such calculations is that the compound was present at 0.1 µg/l in the aqueous medium, when in fact it may have been present at considerably lower concentration levels.

Because of these problems, bioaccumulation factors have been addressed from bcth perspectives. Where possible, bioaccumulation factors have been calculated for compounds which were identified in tissue extracts, but which were either not present at detectable levels in test solutions or detected too infrequently to establish mean concentration levels.

Over the course of the adult California halibut experiment, two aromatic hydrocarbons, mesitylene (1,3,5-trimethyl benzene) and naphthalene, were detected with sufficient frequency to calculate mean concentration levels. Where possible, bioaccumulation factors for these compounds have been calculated for each of the halibut tissues. These data, which are 're-sented in Table 11-43, are from the high exposure level experimental regime. As evidenced by this table, bioaccumulation factors for mesitylene ranged from 12 in muscle tissue to 1598 in gill tissue. Naphthalene was not observed to bioaccumulate as readily, exhibiting a bioaccumulation factor range of 39 in muscle tissue to 214 in gill tissue. Neither compound was observed to accumulate in digestive, eye or gonadal tissue.

Table II-44 presents petrogenic hydrocarbon tissue levels and estimated bioaccumulation factors for individual compounds which were not routinely detected in the aqueous test solutions. The data in this table are categorized in the same manner as the tissue burden data in previous tables.

Table II-43. Mesitylene and naphthalene bioaccumulation factors for adult California halibut target tissues.

Tissue	Mesitylene (1,3,5-trimethylbenzene) Mean Exposure Level (µg/l)[a]	Mean Tissue Burden (ng/g)[b]	Bioaccumulation Factor	Naphthalene Mean Exposure Level (µg/l)	Mean Tissue Burden (rig/g)	Bioaccumulation Factor
Gonad	0.65	ND		0.84	ND	-
Eye	0.65	NO		0.84	ND	-
Muscle (Composite)	0.65	8	12	0.84	37	44
Muscle (individual)	0,65	39	60	0.84	33	39
Liver	0.65	542	834	0.84	NO	
Gill	0.65	1039	1598	0,84	180	214
Digestive Tract	0.65	ND		0.84	NO	

NO = Not detected in tissue extracts.

a Data are from the high exposure level test Solution.

b Data are from organisms exposed to the high level test solution.

Each category is composed of the parent hydrocarbon and its alkyl-substituted homologs. Petrogenic hydrocarbon tissue burden levels are presented as concentration ranges for the individual compounds identified in each category. Mesitylene and naphthalene have been omitted from the ranges presented, since more accurate bioaccumulation factors have been calculated for these compounds previously. Estimated bioaccumulation factor ranges in each category were calculated under the assumption that the mean aqueous exposure level was 0.1 µg/l for each of the individual hydrocarbons. This assumption is based on the knowledge that average aqueous detection limits were roughly 0.1 µg/l for aromatic hydrocarbons of the type identified in tissue extracts.

Estimated bioaccumulation factors for alkyl-substituted benzenes ranged from 290 in muscle tissues to over 6000 in gill tissues. Naphthalene derivatives exhibited estimated bioaccumulation factors ranging from 200 in muscle tissues to over 12000 in liver samples. Thiophene/furan derivatives were identified less frequently, and estimated bioaccumulation factors for these compounds ranged from 160 to 1440. Phenanthrene and its alkyl-

Table H-44. Estimated bioaccumulation factor ranges for petrogenic hydrocarbons in adult California halibut tissues.

Tissue	Benzenes Tissue Burdens	BF[b]	Naphthalenes Tissue Burdens	BF[b]	Thiophenes/Furans Tissue Burden[a]	BF[b]	Phenanthrenes Tissue Burden[a]	BF[b]
Gonad	87	870	100-165	1000-1650	ND		NO	
Eye	NO		69-71	690-710	ND		ND	
Muscle [Composite)	28-58	280-580	20-78	200-780	ND		NO	
Muscle (individual)	36	360	61-73	610-730	16	160	ND	-
Liver	190-399	1900-3990	689-1201	6890-12010	ND		NO	
Gill	80-608	800-6080	47-1197	470-11970	144	1440	ND	-
Digestive Tract	133-494	1330-4940	108-146	1080-1460	32	320	NO	

NO = Not detected in tissue extracts

a Data represent the range of mean concentrations (ng/g dry weight) for individual hydrocarbons identified in tissue extracts from organisms exposed to the hign level test solution.

b BF = estimated bioaccumulation factor ranges, assuming a mean aqueous exposure level of 0.1 µg/l for Individual hydrocarbons in each category (mesitylene and naphthalene are not included in the ranges).

substituted homologs were rtot detected at sufficient levels in any of the halibut tissues to permit estimation of bioaccumulation factors. As Table II-44 indicates, the greatest tissue burdens, and resultant bioaccumulation factors, were observed in the digestive tract, gill and liver.

3) Survivorship. All adult P. californicus survived the first three weeks of exposure in the control and three WSF exposure regimes. Initial mortality of adult halibut occurred during the fourth week in the high concentration (Figure 18) with cumulative survivorship declining to 86.7% by the end of that week. Chi-square analysis indicated the decline was not significant (Table 11-45].

Halibut survivorship decreased markedly in the high concentration between weeks 5 and 7 (Figure 11-18). Initial mortality also occurred in the medium and low concentration regimes during this same period. Following five weeks of exposure, Chi-square analysis indicated cumulative survivorship was significantly lower in the high concentration (26.7%) than in either the control (100.0%), low (100.0%), or medium (93.3%) concentration exposure regimes (Table II-45). At the end of the seventh week all fish in the high concentration tanks had died (Figure Ii-18). Chi-square analysis demonstrated that cumulative survivorship in the high concentration (0.0%) was significantly lower than in the control, low, or medium exposure regimes (Table II-45). Despite qualitative differences in survivorship between the control,

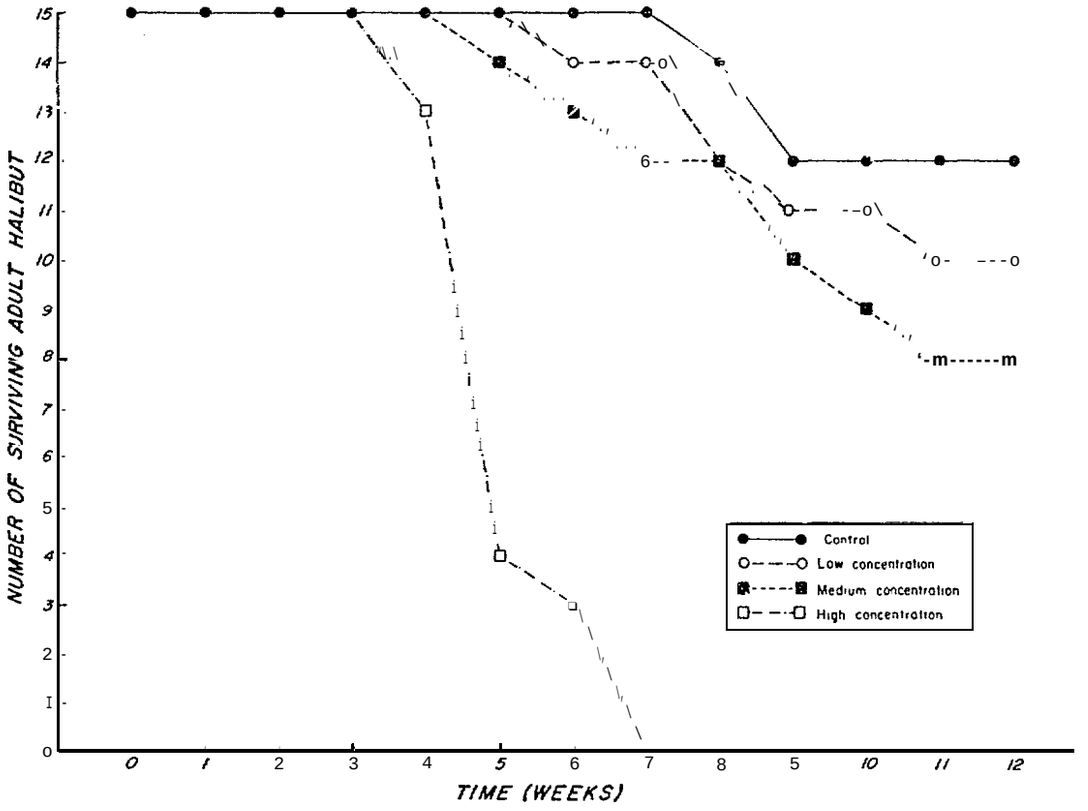


Figure II-18. Cumulative weekly survivorship of adult California halibut.

Table II-45. Cumulative survivorship (Ye) of adult California halibut by week.

Week	Cumulative Survivorship(%) Control	Low	Medium	High
1	100.0	100.0	100.0	100.0
2	100.0	100.0	100.0	100.0
3	100.0	100.0	100.0	100.0
4	100.0	100.0	100.0	86.7
5[a]	100.0[c]	100.0[c]	93.3[c]	26.7[b]
6[a]	100.0[c]	93.3[c]	86.7[c]	20.0[b]
7[a]	100.0[c]	93.3[c]	80.0[c]	0.0[b]
8[a]	93.3[c]	80.0[c]	80.0[c]	0.0[b]
9[a]	80.0[c]	73.3[c]	66.7[c]	0.0[b]
10[a]	80.0[c]	73.3[c]	60.0[c]	0.0[b]
11[a]	80.0[c]	66.7[c]	53.3[c]	0.0[b]
12[a]	80.0[c]	66.7[c]	53.3[c]	0.0[b]

[a] Adult survivorship significantly different ($p < 0.05$) among groups; Chi-square analysis.

[b,c] Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric anal og to SNK **multiple comparison test. Groups with same letter not significantly different.**

low and medium concentrations, no significant difference in cumulative survivor ship was statistically evident.

Gradual mortality occurred in the control, low, and medium concentration regimes between the eighth week and termination of the experiment (Figure 11-18). No significant difference in cumulative survivorship was demonstrable between the control, low, and medium concentration regimes over this period.

Cumulative survivorship ranged from 80.0% in the control to 0% in the high concentration regime upon termination of the experiment (week 12). Chi-square analysis based on cumulative survivorship in each regime at the conclusion of the experiment indicated there was a significant difference in halibut mortality (Table 11-45). Subsequent Chi-square analysis demonstrated that survivorship in the high concentration regime was statistically lower than in the control," low, or medium concentrations. No significant difference in cumulative survivorship was demonstrated between the control, low, and medium concentration exposure regimes.

4) Condition. The total weight of individual California halibut was determined at the beginning of the adult experiment and upon the death of each individual fish or at termination of the experiment. Weights of individual fish were not determined at intermediate times during the 12-week exposure period in order to minimize fish handling and stress. Because of this experimental design, the period of test solution exposure, as well as the associated weight changes of individual fish varied considerably (data appendix).

Most fish in the control and WSF exposure regimes lost weight during the experiment, although a small percentage of fish exhibited a weight gain (data appendix). The mean percentage weight change of test fish ranged from -6.1% (i.e. minus indicates weight loss) in the high concentration to -15.9% in the medium concentration (Table II-46). When the mean percentage weight loss was standardized by the number of exposure days (i.e. mean percent weight change per exposure day), little difference in weight loss was evident for fish in either the control or test solution exposure regimes (Table II-46).

To eliminate the variability in weight loss due to different exposure periods, a statistical comparison was made of the percentage weight loss for only those individual halibut that survived the full experimental period (e.g. 12 weeks). Since no high concentration fish survived beyond seven weeks, this analysis was based only on fish from the control, low, and medium concentration exposure regimes. The mean percent weight change for these fish ranged from -14.3% in the low concentration to -15.2% in the medium concentration (Table 11-47). A K-W ANOVA failed to demonstrate a significant difference in weight change among fish surviving the entire experimental period in the control, low, and medium concentration regimes.

Table II-46. Mean percent weight change and mean percent weight change per exposure day for adult California halibut (Paralichthys californicus).

Concentration	Mean % #eight Change	Mean % Weight Change/Exposure Day
Control	-15.4	-0.22
Low	-14.5	-0.23
Medi um	-15.9	-0.28
High	- 6.1	-0.21

Table II-47. Mean percent weight change for California halibut dying before (pre-termination) and at termination of the experiment.

Concentration	Pre-termi ndti ona	Termination[b, c]
Cent rol	-18.2 (3)	-14.5 (12)
Low	-15.2 (5)	-14.3 (lo)
Medium	-16.7 (7)	-15.2 (8)

a Halibut that died prior to terminati On of experiment.

b Halibut that survived until termination of experiment.

c no significant difference in weight loss ($p > 0.05$); K-W ANOVA.

5) Histopathology. Adult California halibut which died in the high concentration exposure regime were often not discovered for 1 to 8 hrs after death (e.g. when death occurred during the night) resulting in considerable postmortem autolysis of tissues. As a result, very few halibut from the high concentration exposure regime could be histologically evaluated. To minimize this problem in the control, low, and medium concentration regimes, fish were necropsied soon after death and at termination of the experiment.

The results of histological studies on adult Paralichthys californicus are discussed below and summarized in Table II-48. Liver tissues of most test solution-exposed fish exhibited an obvious increase in the size and number of melanin microphage centers (Plates II-6a-d). Melanin microphage centers (MMCs) are groups of macrophages containing melanin which are located between the sinusoids and liver cords, Fish exposed to WSF test solution concentrations also showed reduced cytoplasmic glycogen vacuolation, which was detectable by application of the Periodic Acid Schiff (PAS) test. Minimal increase in the number of cytoplasmic fat vacuoles (fatty change or fatty degeneration) was observed in some livers, but there was no difference between WSF-exposed and control fish. Livers of some low concentration fish contained necrotic cells and hyaline acidophilic bodies (Plate II-6c). This definitive evidence of hepatocellular injury was not observed in any control fish.

Table II-48. **Table II-48. Histopathology summary for adult California halibut tissue.**

Tissue	Histopathological Condition	Frequency of Condition — Concentrations: Medium	Low	Control
Liver	Increase size and number of MMC.	7/14	9/14	2/13
	Glycogen depletion.	7/14	9/14	0/1 3
	Increased cytoplasmic fat vacuoles	0/14	1/14	2/1 3
	Hyaline acidophilic bodies.	0/14	3/14	0/13
Pancreas	No significant change in exocrine or endocrine pancreas.	-	-	
Gill	Hyperplasia of epitheliums.	9/14	8/14	0/13
	Fibrosis and/or cystic change.	2/14	3/14	0/1 3
	Microaneurisms.	6/14	3/14	0/13
Digestive Tract	No significant pathology noted.			
Testis	No significant pathology noted.			
Ova ry	No significant pathology noted.			
Eye	No significant pathology noted.			
Skeletal Muscle	No significant pathology noted.			
Peripheral Blood Cells	No significant pathology noted.			

The exocrine portions of the pancreas were normal and composed of well-formed acini lined by epithelial cells possessing basophilic cytoplasm and eosinophilic zymogen granules within the cell apices. The pancreatic duct system was normal in both test solution-exposed and control halibut. Islets of Langerhans also exhibited normal cell populations with no significant change due to WSF exposure.

Gill tissue of WSF-exposed fish exhibited pathological changes not found in control fish. Clubbing of the secondary lamellae tips was observed, due to epithelial cell proliferation and fibrosis. Many secondary lamellae were thickened and shortened, chronically inflamed and fibrotic. Adhesions also formed between adjacent secondary lamellae, resulting in cyst formation (Plate II-7a-c). In addition, microaneurisms were observed at the tips of secondary lamellae in a number of fish (Plate II-8). In general, gill damage was more severe in fish exposed to the medium rather than the low concentration exposure regime. Evidence of gill damage was absent from the control group (Plate II-9).

The intestinal tract epitheliums was normal appearing and exhibited no metaplastic changes, ulceration, or atypicality suggesting neoplasia. In many instances, helminths (i.e. acanthocephalans, trematodes, nematodes, or cestodes) were attached to intestinal mucosa or found within the intestinal wall. There was no clear difference in this regard between control or WSF-exposed fish. Infiltration of the intestinal wall by chronic inflammatory cells (including lymphocytes and histiocytes) varied among fish in the different exposure regimes; however, there was no distinguishable difference between control and WSF-exposed fish.

Histological sections of the eye also exhibited no significant pathology. Nerve cells at various levels in the retina were enumerated, but no differences were found between control and WSF-exposed fish. There was considerable variation in the number of ganglion cells of the inner layer in various specimens; however, this was not related to the WSF-exposure regime.

Sections of skeletal muscle likewise revealed no significant pathology. Occasional degenerating muscle fibers were observed in both experimental and control fish. These were vacuolated and partially invaded by histiocytes. Peripheral blood cells observed within vascular spaces in the tissue sections showed no unusual differences between control or WSF-exposed fish.

6) Behavior. Because of the relative inactivity of flatfishes, assessment of California halibut activity was limited to observations on their visual (food) and physical (touch) responsiveness to stimuli, as well as eye, gill, and fin movements. Body coloration and posture proved to be the two most important indicators of fish health and physical condition. Halibut in poor health darkened in color (from a light gray or brown to a dark gray), and often assumed a posture characterized by an arched spinal column. The latter indicator was so reliable of pending death that halibut exhibiting this posture were usually dead within 24 hrs.

Throughout the first two weeks of the experiment, all fish appeared healthy and normal. By week 3, however, the first signs of stress became apparent. The most common sign was a distinct darkening of halibut body coloration in the high concentration. In general, the responses of these

Plate 11-6(a-d). Adult *Paralichthys californicus* liver from low concentration regime.

a. Note two melanin microphage centers, each of which is indicated by a group of three arrows. Portal tract (left, upper) has dark-staining pancreatic acinar tissue and clearly visible branches of portal vein (V) and hepatic artery (H).

b. Hepatocytes have fine cytoplasmic vacuolation, mostly due to glycogen, with some larger vacuoles due to fat. Central vein (CV). Portal tract (P) has associated pancreatic acinar tissue (very dark-staining).

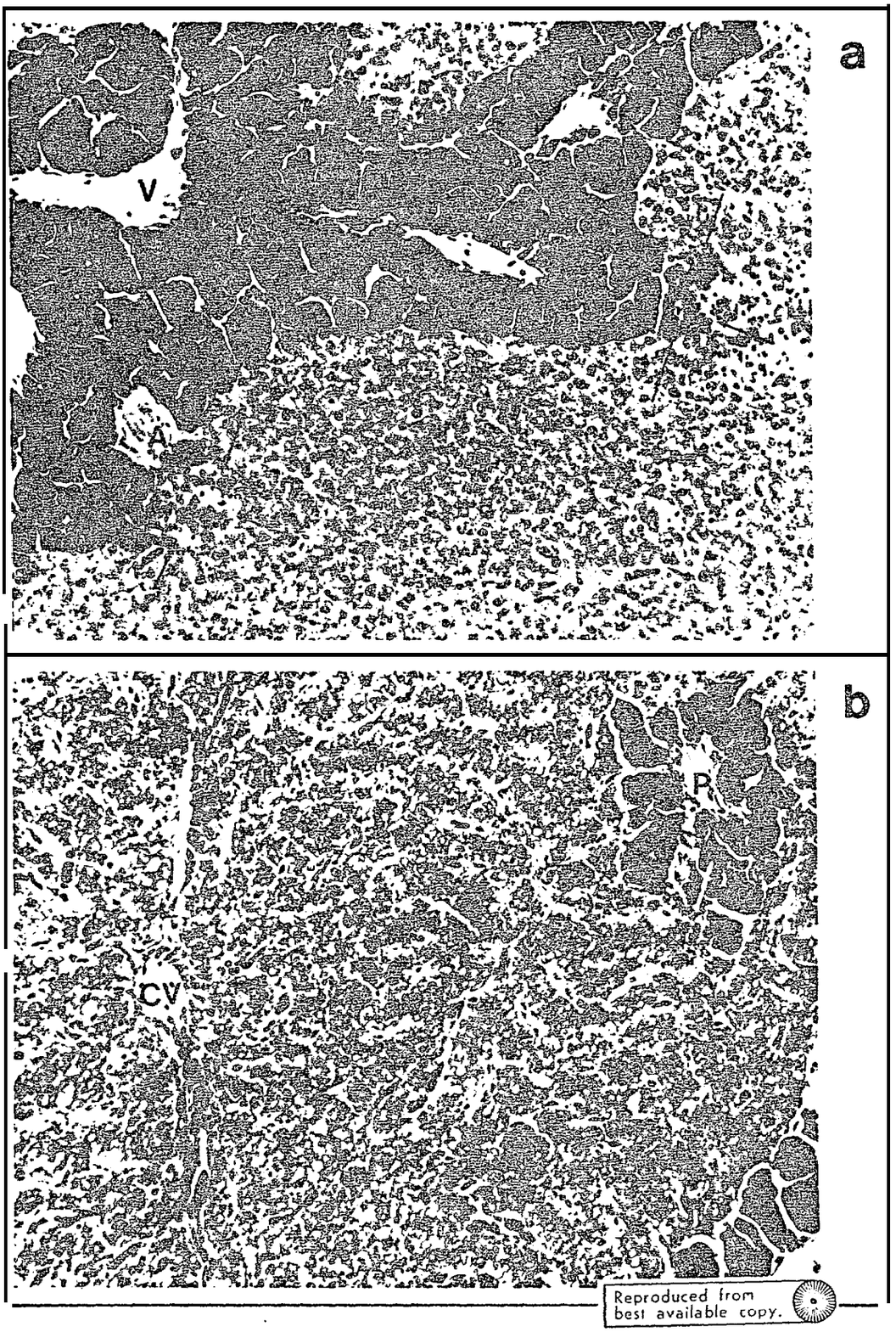


Plate II-6(a-d). (Cent)

c* Two melanin microphage centers (MMC) are clearly seen in the mid-lower picture. Acidophilic hyaline body (arrow). (X325).

d. Melanin microphage center (MMC). (X325).

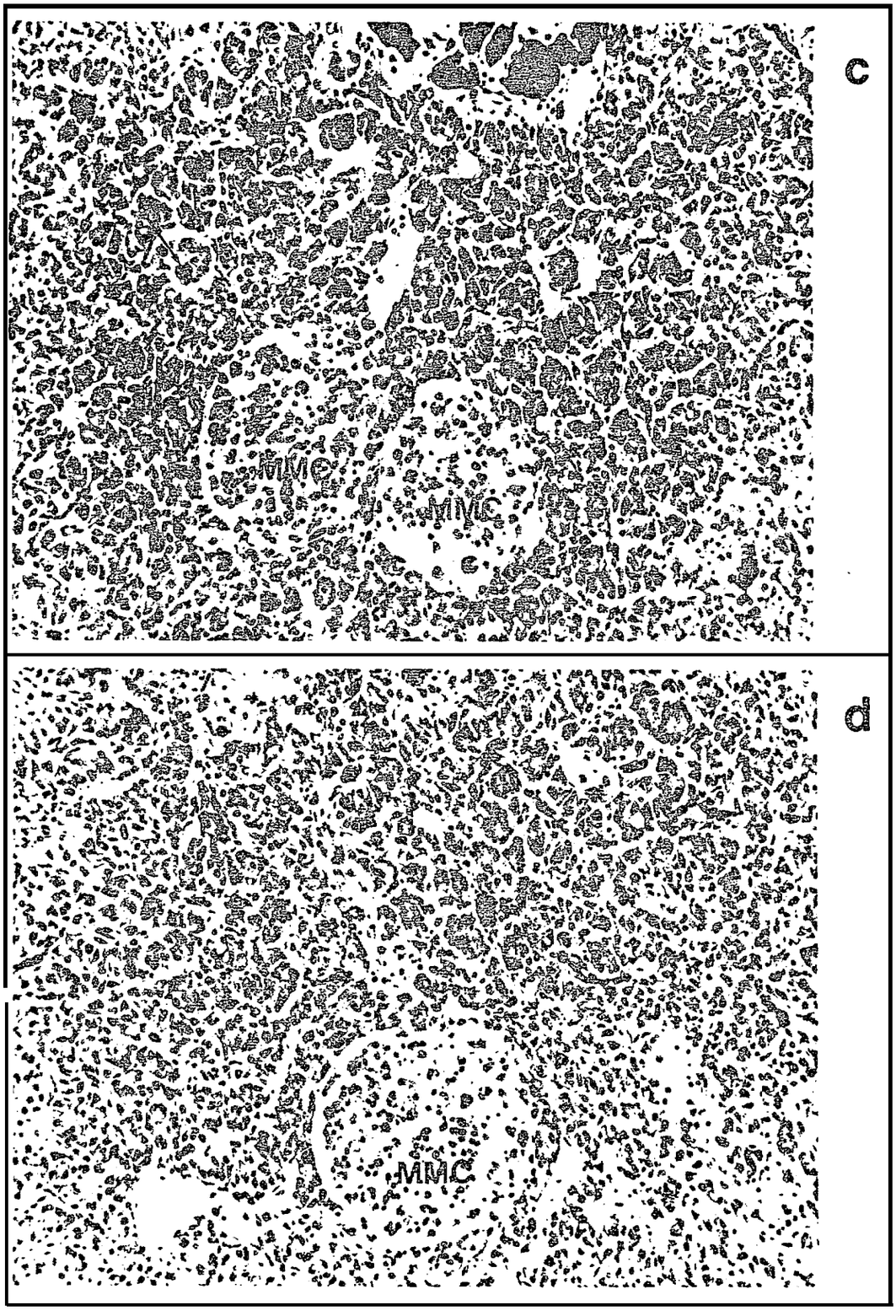

c
MMC
d
MMC

Plate 11-7(a-c). Adult Paralichthys californicus gill from medium concentration regime.

a. There is marked epithelial hyperplasia with adhesions between adjacent secondary lamellae, inflammation and fibrous obliteration of secondary lamellae with resulting epithelial cyst formation (Cy). Cartilage (C) of primary lamella. Relatively normal secondary lamellae (S). (X325).

b. Cyst (Cy) in fused secondary lamellae. Cartilage (C) of primary lamellae. (X325).

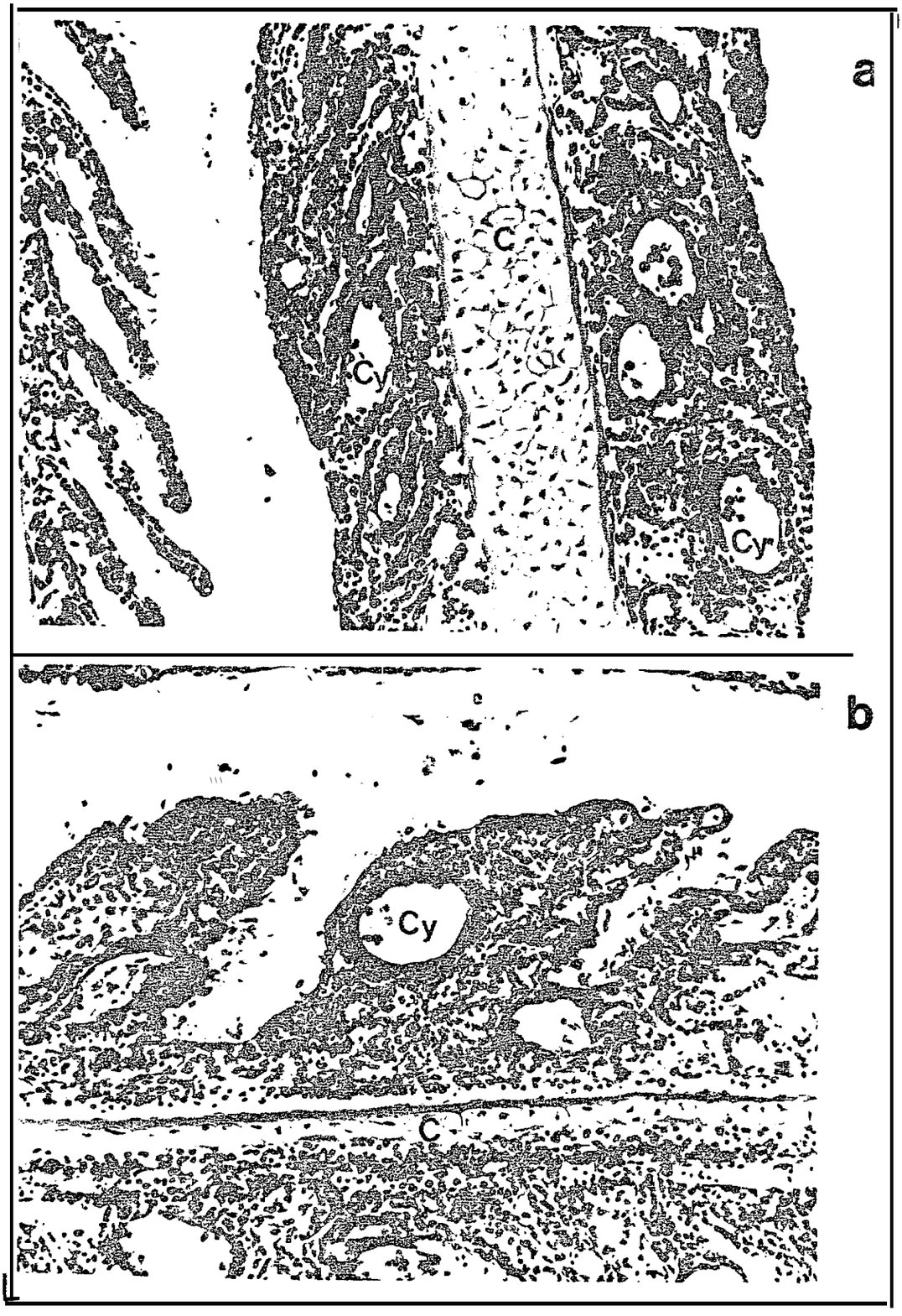

a
Cy
C
Cy
b
Cy
C

Plate 11-7(a-c). (Cent)

c. Note chronic inflammation (Arrows), goblet cell hyperplasia (G), and loss of secondary lamellae above. Secondary lamellae below are normal. Cartilage (C). (X325).

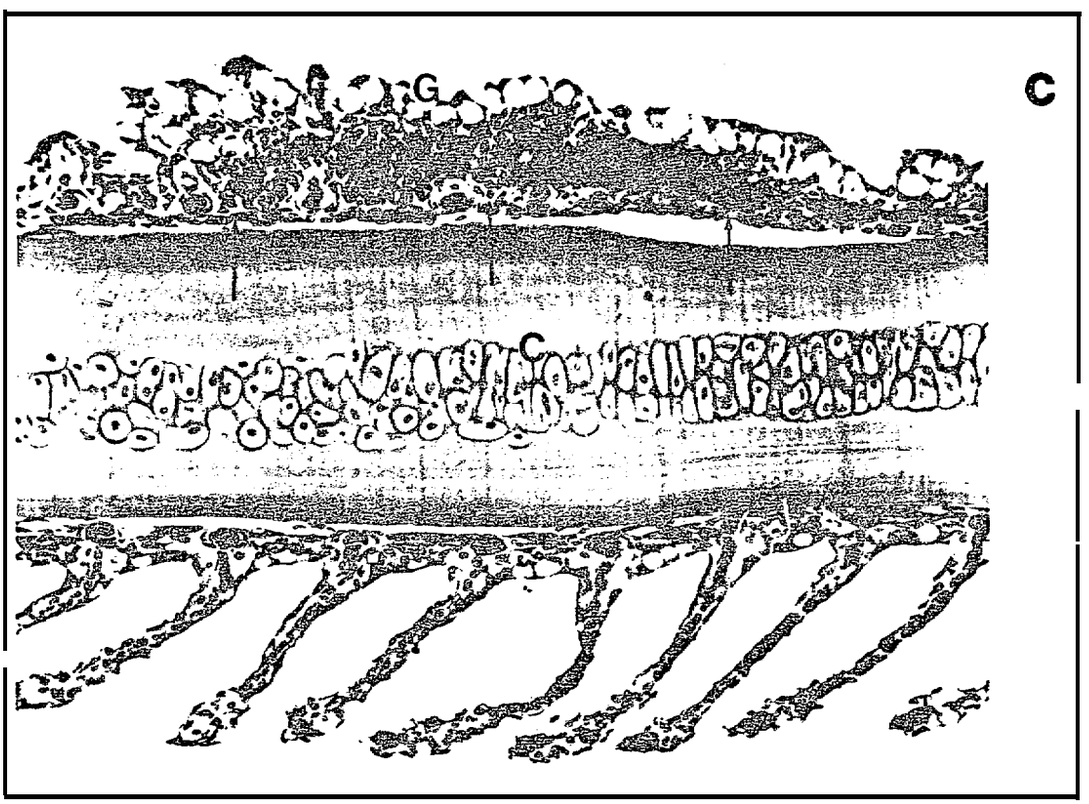
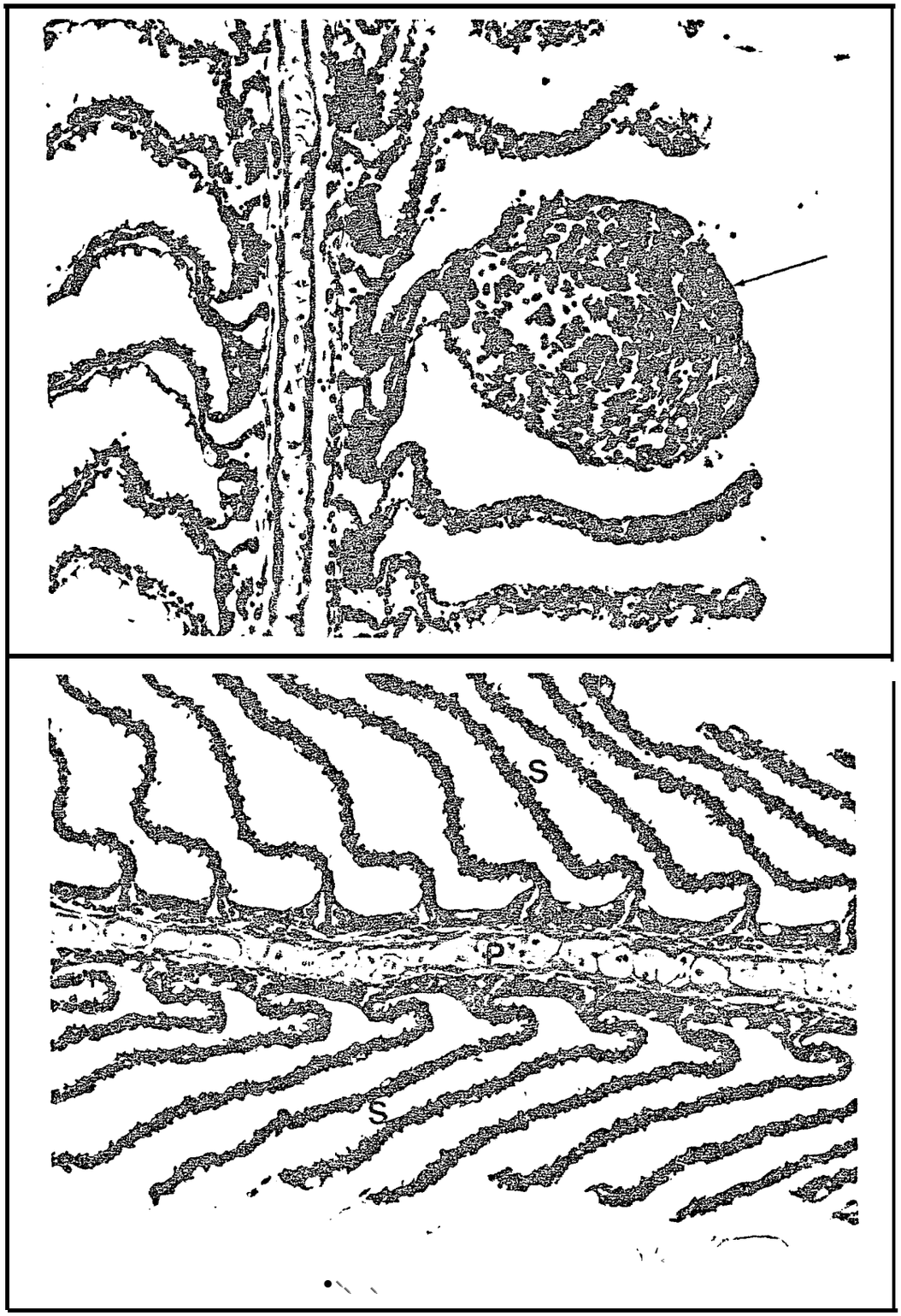

G
C
C

Plate II-8. Adult Paralichthys californicus gill from low concentration regime. Microaneurism of secondary lamella (arrow). The microaneurism is greatly distended with blood. (X325).

Plate II-9. Adult Paralichthys californicus gill from control regime. One primary lamella (P) with many secondary lamellae (S). (X325).



b

fish to stimuli appeared to be more sluggish than for fish in the lower concentrations. Initial fish mortality occurred during week 3. One individual in the high concentration was observed in a convulsive posture with an arched spine and died the following day.

By week 4 of the experiment, halibut in the high concentration exhibited a variety of abnormal physical and behavioral changes indicative of severe stress. Notable examples included darkening of body color, arching of spine, mouth gaping, fin hemorrhaging, scale loss, abrasions, excessive mucus secretion, rigid curling of fins, and surface swimming.

Mortality in the high concentration tanks continued throughout weeks 5 and 6. More significantly, however, initial mortality in the medium concentration occurred during this period. The only indication of stress in the medium concentration was hemorrhaging along the caudal fin. Also noteworthy during this period was the onset of a parasitic outbreak. Observations were hindered for several days due to a heavy storm (turbid, silty seawater). When water clarity permitted, large numbers of parasitic leeches were observed on fish in all tanks. Because the infestation was so widespread, parasites were most likely introduced either from the source seawater, the food supply (live Callianassa sp.), or from Mytilus californianus (mussel experiments were run concurrently in the same tanks as the halibut experiment). The initial infestation was highest in the medium concentration, less severe in the high and low concentrations, and least severe in the controls.

Formalin treatments were used to eradicate the parasite infestation. During weeks 5 and 6, all halibut were subjected to two formalin baths at a dose of 80 mg/l for 30 minutes (van Duijn 1973). The treatments proved moderately successful and greatly reduced the parasite load. Particularly in the control tanks, individual fish appeared more active and displayed relatively normal coloration following treatment.

A second parasitic leech outbreak occurred during week 7 of the experiment. As in the previous outbreak, fish in the experimental tanks were more severely infested than control fish. Symptoms previously associated only with exposure to the test solution (e.g. dark color, arched spines, fin hemorrhaging) began to appear in both NSF-exposed and control fish. By the week 8, it became extremely difficult to discern whether such symptoms resulted from WSF exposure or the parasitic infestation.

A commercial insecticide, Dylox (O,O,-dimethyl -2,2,2-trichloro-1-hydroxyethyl phosphonate), was administered at a dosage of 1 ppm for 24 hrs early in week 8. The Dylox treatments resulted in almost complete eradication of the parasite. Within 24 hrs of treatment, the remaining fish displayed noticeable improvement in both appearance and activity.

The final three weeks of the experiment were characterized by a stabilization in the mortality rate. Only three fish died during this period (two in the medium concentration and one in the low concentration). Although the parasitic infestation persisted throughout the remainder of the experiment, the overall condition of control fishes showed a marked improvement. Control fishes appeared healthier, displayed normal coloration and were more responsive to stimuli. In contrast, low and medium concentration fishes suffered a general degradation in physical condition during the final four weeks of the experiment. This overall degradation in physical condition included 1) skin abrasions that became infected thus exposing large areas of underlying muscle tissue and 2) increased scale loss and mucus secretion.

3. NORTHERN ANCHOVY (Engraulis mordax)

a. Embryo Experiment

1) Hydrocarbon Exposure. Northern anchovy (Engraulis mordax) embryos were exposed to control seawater and three WSF test solution concentrations for approximately 48 hrs. As shown in Table II-49, the high background hydrocarbon levels observed in control waters resulted in very little difference between total hydrocarbon concentrations in control and low level exposure regimes during the embryo study. Control and low concentration hydrocarbon levels (minus diethyltetrahydrofuran) were 22.7 and 23.9 µg/l, respectively. Total hydrocarbon concentration (minus contaminant concentrations) levels in medium and high exposure test solutions were 188 and 367 µg/l, respectively.

2) Hydrocarbon Tissue Burden. Hydrocarbon bioaccumulation by E. mordax embryos exposed to the WSF test solution exposure was indirectly-measured using a radioactive tracer (^{14}C-naphthalene). Mean radioactive tracer uptake by E. mordax embryos was directly proportional to the WSF test solution exposure concentration (Table 11-50; data appendix). Tracer uptake was lowest at the low concentration and highest at the high concentration. Uptake by control embryos was comparable to that found for embryos exposed to the low concentration. A K-W ANOVA based on ranked uptake data demonstrated that radioactive tracer uptake was significantly different p <0.05) in WSF-exposed and control embryos.

Table II-49. Hydrocarbon exposure concentrations for northern anchovy embryo experiment.

Exposure Level	Hydrocarbon Concentration (µg/l)a Volatile Fraction	Extractable Fraction	Total
control	1.0	21.7 [24.7]	22.7 [25.71
Low	2.8	21.1 [23.7]	23.9 [26.5]
Medium	82.2	106 [110]	188 [192]
High	167	200 [210]	367 [377]

a Extractable and total hydrocarbon values are reported corrected for the presence of diethyl tetrahydrofuran and uncorrected values are also presented in brackets.

Table II-50. Mean radioactive tracer uptake (counts/minute/embryo) by Engraulis mordax embryos. Standard errors are shown in parentheses.

Concentration	Mean Counts Per Sample[a]	Standard Error
Control	5.69[b]	0.25
Low	5.52[b]	0.08
Medium	6.39[c]	0.41
High	8.92[d]	0.35

a Tracer uptake significant different (p <0.05) among groups; K-W ANOVA.

b,c,d Groups designated by different letters significantly different (p <0.05) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

To determine which specific test solution concentrations resulted in significant differences in radiotracer uptake, a non-parametric analog of the SNK multiple comparison procedure based on rank sums was employed. Because of unequal sample sizes among the control and test solution concentrations, it was necessary to randomly eliminate one replicate each from the control, low and medium concentrations prior to using the test. Results of multiple comparison testing (Table II-50) indicated that embryo radioactive tracer uptake was: 1) significantly greater (p <0.05) in the high concentration than in the medium, low or control; and 2) significantly greater (p <0.05) in the medium concentration than in the low or control. Uptake by embryos in the low concentration, however, was not significantly different from control embryos.

3) Embryo Mortality and Hatching Success. Mortality of E. mordax embryos ranged between 62.6% and 68.4% in the low and medium concentration

Table II-51. Percentage mortality (mean and range) of Engraulis mordax embryos in control and test solution exposure regimes.

Concentrate ion	No. of Repl icates	% Mortal i ty Mea n	Range
Cent rol	6	60.4	54.6 -66.6
Low	6	62.6	54.6 -68.0
Medium	6	68.4	56.0 -80.0
High	6	68.1	60.0 -76.0

Table II-52. Mean number of dead Engraulis mordax embryos per replicate. Standard errors are shown in parentheses.

Concentration	Initial Number of Embryos per Replicate	Mean Number of Oead Embryos per Replicate
Control	75	45.33 (1.26)
Low	75	47.00 (1.57)
Medium	75	51.33 (2.90)
High	75	51.17 (1.96)

[a] Number of dead embryos not significantly different ($p > 0.05$); K-H ANOVA.

exposure regimes, respectively (Table 11-51: data appendix). and tended to increase with increasing WSF exposure concentration. The range of mortality observed among individual replicates in the low, medium and high concentrations, however, was 54.6% to 80.0% (Table 11-51). Percent mortality in the control replicates ranged between 54.6 and 66.6% with a mean of 60.4%. In general, embryo mortality was more variable among replicates in the medium and high concentrations (56.0 to 80.0%) than in either the control or low concentration (54.6 to 68.0%).

The mean number of dead embryos per replicate was used as the measure of embryo mortality for statistical evaluation, since the initial number of fertilized eggs placed in each replicate was equal (75 eggs) (Table II-52). A K-W ANOVA based on ranked mortality data revealed that mortality was not significantly different for WSF-exposed and control embryos.

Hatching success (i.e. percentage of embryos successfully hatching) of E. mordax embryos exposed to the WSF test solution concentrations ranged from 54.0% in the medium concentration to 75.0% in the low concentration (Table II-53). Hatching success was highest (86.0%) for control embryos. Chi-square analysis indicated that batching success of WSF-exposed and control embryos was significantly different ($p < 0.05$). Subdivision of the Chi-square contingency table following Zar (1974; p. 65) demonstrated that hatching success of E. mordax embryos was significantly greater in the control than in either the medium or high concentration regimes. Control embryo hatching success, however, was not significantly greater than in the low concentration. Further analysis indicated no significant difference in embryo hatching success between the medium and high concentration exposure regimes.

Table 11-53 . Number 01 successfully and unsuccessfully hatched Engraulis mordax embryos in each test solution exposure-e. Embryo totals are summed over ail replicates within a treatment. Proportions of embryos in each category are shown in parentheses.

Concentration	Total Number of Eggs	Embryos Successful in Hatching[a]	Embryos Not Successful in Hatching
Control	88	76 (0.86)[b]	12 (0.14)
Low	78	59 (0.75)[b]	19 (0.251
Medium	52	28 (0.54)[c]	24 (0.46)
High	50	31 (0.62)[c]	19 (0.38)

[a] Hatching success significantly different ($p < 0.05$) among groups; Chi-square analysis.

[b,c] Groups designated by different letters significantly different ($p < 0.05$) fror all other groups; based on subdivision of Chi-square contingency table. Groups with same letter not significantly different.

4) Histopathology. Histological examination of selected organs in embryos was not feasible, since an extensive research and development effort was needed.

b. Larval Experiment .

1) Hydrocarbon Exposure. Northern anchovy larvae were exposed to control seawater and three concentrations of

WSF test solution for up to 14 days. Table II-54 summarizes the hydrocarbon concentration levels for each of the aqueous exposure concentrations used in the larval experiment. Control and low exposure level hydrocarbon values were statistically different at the 95% significance level (Student's t-test). The mean hydrocarbon concentration in low exposure level aquaria was 29.5 µg/l when corrected for the background contribution. Mean total hydrocarbon levels for medium and high exposure concentrations were 85 and 384 µg/l, respectively.

2) Hydrocarbon Tissue Burden. Radioactive tracer uptake by larval E. mordax was determined for control and WSF-exposed larvae after 4, 7 and 14 days of test solution exposure (Table II-55). Because of extremely high mortality in the high concentration only a single estimate of tracer uptake could be obtained on day 4 of the experiment. Comparison with a parallel static control indicated that larval tracer uptake in the high concentration was nearly two times greater than for larvae in the control (Table II-55).

Table II-54. Hydrocarbon exposure concentrations for northern anchovy larval experiment.

Exposure Level	Hydrocarbon Concentration (µg/l)[a] Volatile Fraction	Extractable Fraction[b]	Total[c]
Control	1.7± 2.2	12.6± 1.7 [19.3±4.9]	14.4±0.8 [21.0±4.0]
Low	4.3± 4.2	25.5± 7.2 [34.7±7.6]	29.9± 5.3 [39.0±5.7]
Medium	32.1±19.2	53.3 [68.31	85. 0 [100]
High	1843131	200 [210]	384 [394]

a Where appropriate, data are reported as the mean ±1 standard deviation.

b Extractable hydrocarbon values are reported corrected for the presence of diethyl tetrahydrofuran and uncorrected values are also presented in brackets.

c Total hydrocarbon values represent the sum of the average daily volatile fraction plus the weekly extractable fraction. Values are also reported both corrected and uncorrected for diethyl tetrahydrofuran.

Table 11-55. Mean radioactive tracer uptake per larva (total counts) by Engraulis mordax larvae of 4, 7, and 14 days. Standard errors are shown in parentheses.

Concentration	Exposure Period (days) 4	7b	14
Control	15.7[a]	8.26[c] (0.35)	13.20 [0.32)
Low		7.81[c] (0.40)	13.30[e] -
Medium		9.45[d] (0.35)	- -
High	28.05[e]	- -	-

a Static control.

b Tracer uptake significantly different ($p < 0.05$) among groups; K-W AlJOVA.

c,d Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

e Based on single estimate of tracer uptake.

Radioactive tracer uptake on day 7 was determined for three replicates from the control, low and medium concentrations, respectively. Mean tracer uptake ranged between 7.8 and 9.5 counts/minute/larva in the low and medium concentrations, respectively (Table 11-55). Mean tracer uptake in the control was comparable to that found in the low concentration. A K-W ANOVA based on ranked uptake data showed that larval tracer uptake was significantly different for control and WSF-exposed larvae. Multiple comparison testing indicated there was no significant difference in tracer uptake between control and low concentration larvae. Larval tracer uptake in the medium concentration was significantly greater than in either the control or low concentration.

Since only a single estimate of tracer uptake was obtained for " larvae from the low concentration at day 14, no statistical comparison was possible with the control. However, a qualitative comparison of uptake in the control and low concentration test solution regimes indicated little difference in uptake (Table II-55).

3) Survivorship. Survivorship of larval E. mordax was extremely low in the control and all test solution exposure regimes; however, there was a clear inverse relationship between larval survivorship and exposure concentration (Figure 11-19, Table II-56; data appendix). For example, larvae exposed to the high and medium concentrations survived only 4 and 10 days, respectively, whereas control and low concentration larvae survived for the full 14 day experiment.

Three replicate containers were randomly terminated from each of the control, low and medium concentration test solutions to determine larval mortality on day 7 of the larval experiment. Since the same number of larvae (150) was initially introduced into each replicate, the mean number of surviving larvae per replicate was used for statistical evaluation of survivorship. The mean number of surviving larvae ranged from 10.67 in the medium concentration to 26.33 in the control (Table II-56). A K-W ANOVA indicated that larval survivorship differed significantly ($p < 0.05$) in the control, low, and medium concentration exposure regimes. A SNK multiple comparison test revealed that larval survivorship was significantly greater in the control than in low and medium concentration regimes. There was no significant difference between the number of surviving larvae in the low and medium concentrations.

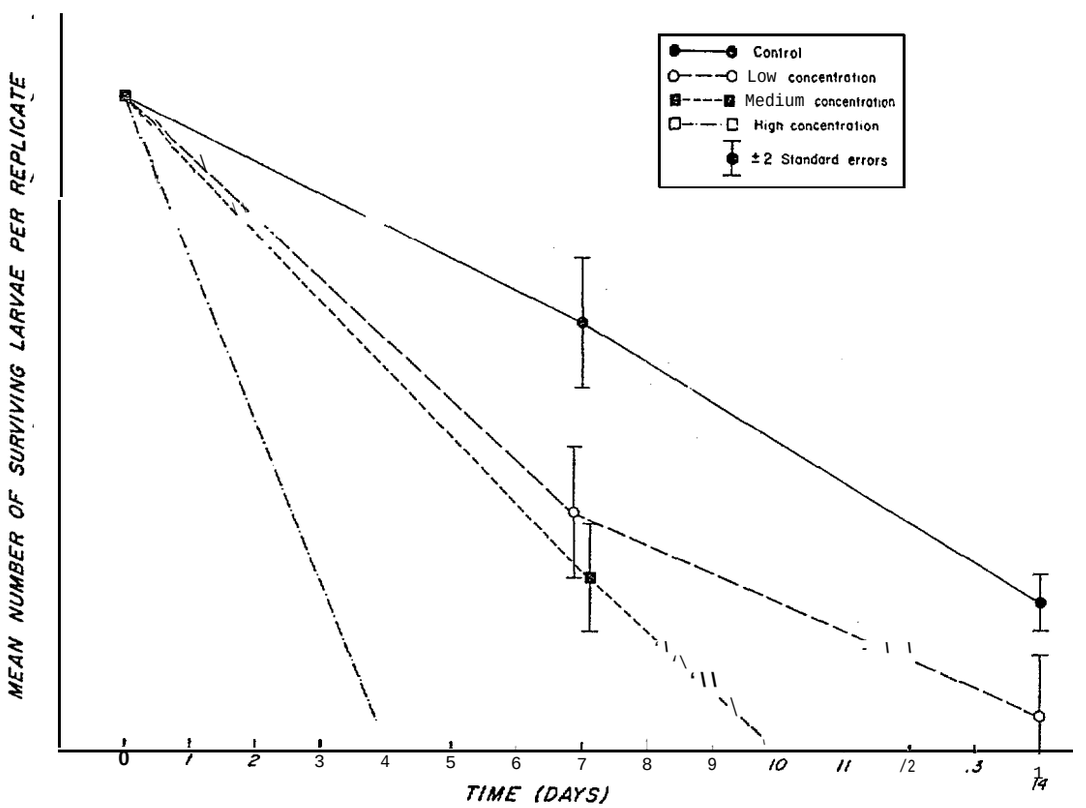


Figure II-19. Mean number of surviving larval northern anchovy following 4,7,10, and 14 days of test solution exposure.

The larval experiment was terminated on day 14 and the three remaining replicates from the control and low concentration exposure regimes were examined for surviving larvae. Mean numbers of surviving larvae were 9.33 and 2.33 in the control and low concentrations, respectively (Table II-56). A Mann-Whitney U-test revealed that survivorship was significantly greater ($p < 0.05$) in the control concentration regime than in the low.

4) Growth. The mean total length (TL) of newly-hatched E. mordax larvae was assumed to be 2.98 mm based on a parallel experiment conducted under static conditions (Table II-57). Following seven days of exposure, the mean total length (TL) of surviving larvae were 4.78 and 5.35 mm in the medium and low concentrations, respectively (Table II-57, Figure 11-20; data appendix). The mean TL of control larvae was 5.39 mm after seven days. A K-W ANOVA demonstrated that larval growth, as measured by mean TL, was significantly different ($p < 0.05$) in the control, low and medium concentration exposure regimes. A non-parametric SNK multiple comparison test, based upon ranked total length data, indicated there was no significant difference in larval growth between the control and low concentration exposure regime. Larval growth in the medium concentration, however, was significantly depressed when compared with growth in both the control and low concentrations.

Table II-56. Mean number of surviving Engraulis mordax larvae at 4, 7, and 14days. Standard errors are shown in parentheses.

Concentration	0[a]	Exposure Period [days] 4	7[b]	14[e]
Control	150	-	26.33[c] (2.02)	9.33 (0.88)
Low	150	-	14.67[d] (2.02]	2.33 (1.85)
Medium	150	-	10.67[d] (1.67)	-
High	150	0.6 (0.481	-	

a Initial number of larvae per replicate.

b Number of surviving larvae significantly different among groups ($p < 0.05$); K-W ANOVA.

c,d Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

e Number of surviving larvae significantly different ($p < 0.05$); Mann-Whitney test.

Table II-57. Mean total length (TL) in mm of surviving Engraulis mordax larvae following 4, 7, anat14days ofexposure. Standard errors are shown in parentheses.

Concentration	0[a]	Exposure Period (days) 7[b]	14[e]
Control	2.98	5.39[c] (0.22)	6.49 (0.33)
Low	2.98	5.35[c] (0.261	5.50 (0.15)
Medium	2.98	4.78[d] (0.21)	-
High	2.98		-

a Mean size at hatching.

b TL of surviving larvae significantly different ($p < 0.05$) among groups; K-W ANOVA.

c,d Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

e TL of surviving larval significantly different ($p < 0.05$); Mann-Whitney test.

Upon termination of the larval experiment (day 14), the mean total length of larvae from the control and low concentration test solutions were 6.49 and 5.50 mm TL, respectively (Table II-57 and data appendix). A Mann-Whitney test indicated growth was significantly reduced ($p < 0.05$) in the low concentration compared with the control. Although larval growth was comparable in both the control and low concentration, a significant divergence in growth occurred by day 14 (Figure 11-20). In particular, there was little growth of larvae in the low concentration between days 7 and 14 of the experiment, whereas the size of control larvae increased by over 20% (Table II-57 and Figure 20).

5) Development. Development of larval E. mordax was assessed after 7 and 14 days of test solution exposure. Several morphological characters including

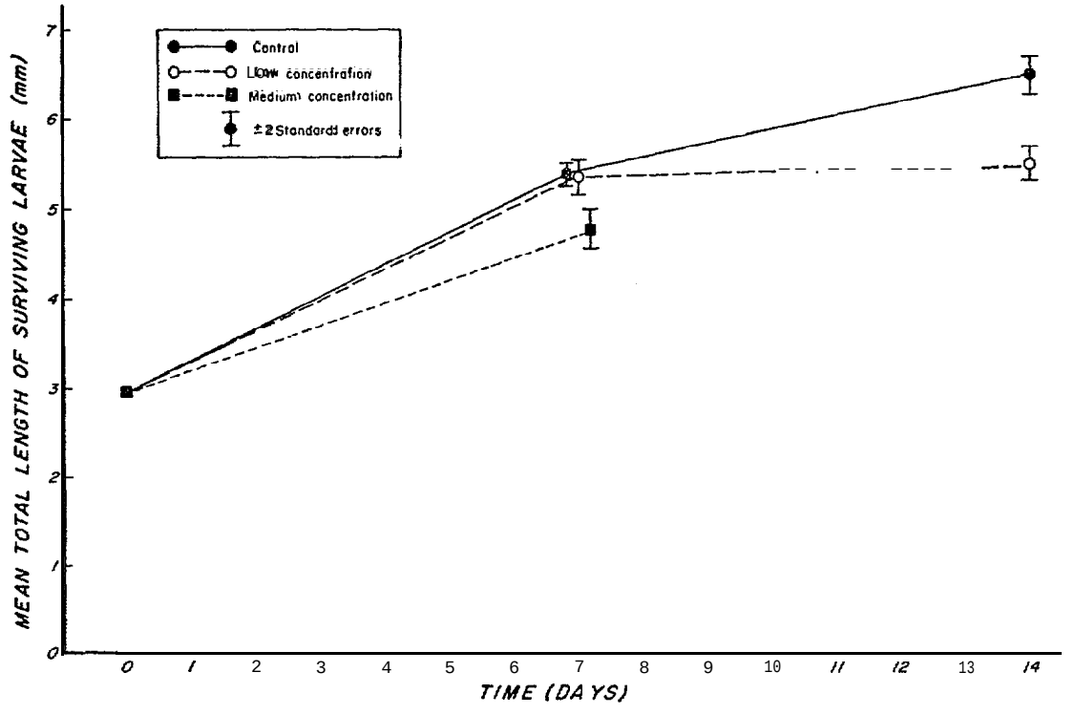


Figure II-20. Mean total length of surviving larval northern anchovy following 7 and 14 days o! test solution exposure.

snout-vent (SV) length, eye diameter (anterior-posterior distance) and eye-to-tip-of-snout (ETS) distance were measured on larvae from the control, low, and medium concentration exposure regimes. Larvae from the high concentration regime had all died by day 4 of the experiment, and were not available for assessment of larval development because of decomposition.

Following seven days of test solution exposure, larvae in the medium concentration were generally less well-developed than larvae from the control or low concentration regimes (Plate 11-10a-c). The head, jaw, and posterior gut (i.e. vent) of medium concentration larvae were particularly less well-developed, with both the S-V length and ETS distance significantly reduced (Table 11-58). The reduced S-V length of medium concentration larvae was correlated with a significant reduction in total length (Table II-57). No medium concentration larvae survived the entire 14-days.

No significant differences in measured morphological characteristics were evident between control and low concentration larvae after 7 days of exposure (Plate 11-10a-c; Table II-58). Low concentration larvae were clearly less well developed (Plate II-ha-b) and smaller (Table II-57) following 14 days of exposure. S-V length, eye diameter, and ETS distance were all significantly reduced (Table II-58) in low concentration larvae compared with controls. Reduced cranial ossification and pectoral fin development also characterized the low concentration 14-day larvae (Plate II-ha-b).

6) Histopathology. Histological examination of selected organs in larval E. mordax was not feasible, since an extensive research and development effort

was needed. However, whole organism mounts were prepared and subjected to microscopic examination. These results are presented in Section 3.a.5, Development.

7) Behavior. Behavioral observations conducted during the first four days of exposure were made both on larvae in the experimental flow-through system and on those in a parallel static system. This procedure was necessary due to difficulty in seeing and locating newly-hatched larvae in the flow-through system.

Newly-hatched larvae were observed congregating at the air/water interface in all WSF exposure regimes. Most were observed to passively float although occasional movement was also noted. This movement consisted of the larvae rapidly "whipping" their tails from side to side. Larvae were spread throughout the water column in all exposure regimes on day 2, although the highest densities still occurred at the air/water interface. By day 3, no larvae were observed in the water column of the high concentration regime. Larvae observed at this level were located on or near the bottom of the test aquaria attempting to swim upwards, but when swimming terminated they would s" . back to the bottom. Larvae in the remaining three exposure regimes were spread throughout the water column; these larvae-spent a majority of their time passively floating with occasional swimming and "striking" movements.

Table II-58. Mean snout-vent (SV) length, eye diameter and ETS distance of Engraulismordax larvae following 7 and 14 days of test solution exposure. Mean based on the measurement of six larvae. Standard errors are shown in parentheses.

Morphological Character	Control	Low	Medium[b]	Highs
		7-day Exposure		
Snout-Vent (SV) Length[d]	2.90[e] (0.04)	2.86[e] (0.05)	2.40[f] (0.05)	
Eye Diameter (ED) Anterior-Posterior[c]	0.24 (0.04)	0.23 (0.03)	0.22 (0.02)	-
Distance From Eye to Tip of Snout (ETS)[d]	0.09[e] [0.021	0.08[e] (0.02)	0.06[f] (0.02)	-
		14-day Exposure		
Snout-Vent (SV) Length[g]	4.09 (0.28)	3.34 (0.071	-	-
Eye Diameter (ED) Anterior-Posterior[g]	0.30 (0.03	0.26 (0.041	-	-
Distance From Eye to Tip of Snout (ETS)[g]	0.12 (0.02)	0.10 [0.02)	-	-

a All high concentration larvae died prior to day 7 of experiment.

b All medium concentration larvae died prior to day 14 of experiment.

c No significant difference ($p > 0.05$) among groups; K-W ANOVA.

d Significant difference ($p < 0.05$) among groups; K-U ANOVA.

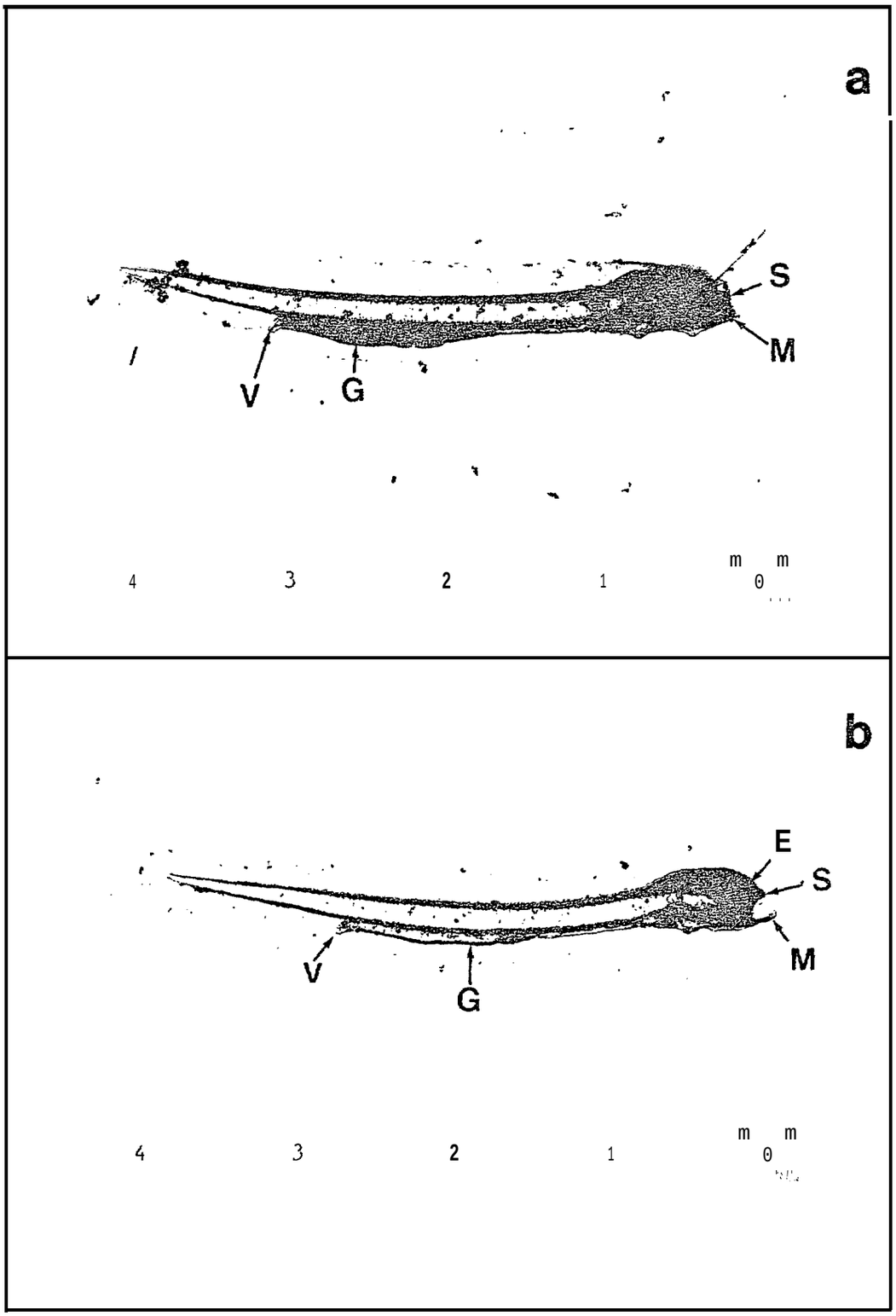
e,f, Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

g Significant difference ($p < 0.05$) among groups; Mann-Whitney test.

Food in the form of Gymnodinium splendens was added to all but the high concentration regime on day 4. Approximately two to three hours after food was added, the dinoflagellate formed dense "patches" at or near the surface of the test aquaria. Larvae in all exposure regimes soon congregated on the outer perimeter of the dinoflagellate "patches" and appeared to feed. Their behavior consisted of a series of short "striking" movements which moved the larvae through the dinoflagellate "patch."

No change in larval behavior at the medium concentration was noted until day 2 at which time larvae were observed to float head down with little movement. Larvae in the control and low concentration still congregated near the food patches. Larval behavior consisted of swimming and striking movements followed by periods of passive floating. On day 6, the rotifer Brachionus plicatilis was added to all culture aquaria. This did not appear to affect the

Plate II-10(a-c). Representative photomicrographs of whole-mounted larval northern anchovy from the control (a), low (b), and medium (c) concentration exposure of regimes following 7 days of exposure. Note the smaller head, shorter snout, smaller eye, and less well-formed vent in the low and medium concentration larvae. Structures labeled as follows: Eye (E), snout (S), mouth (M), vent (V), and gut (G).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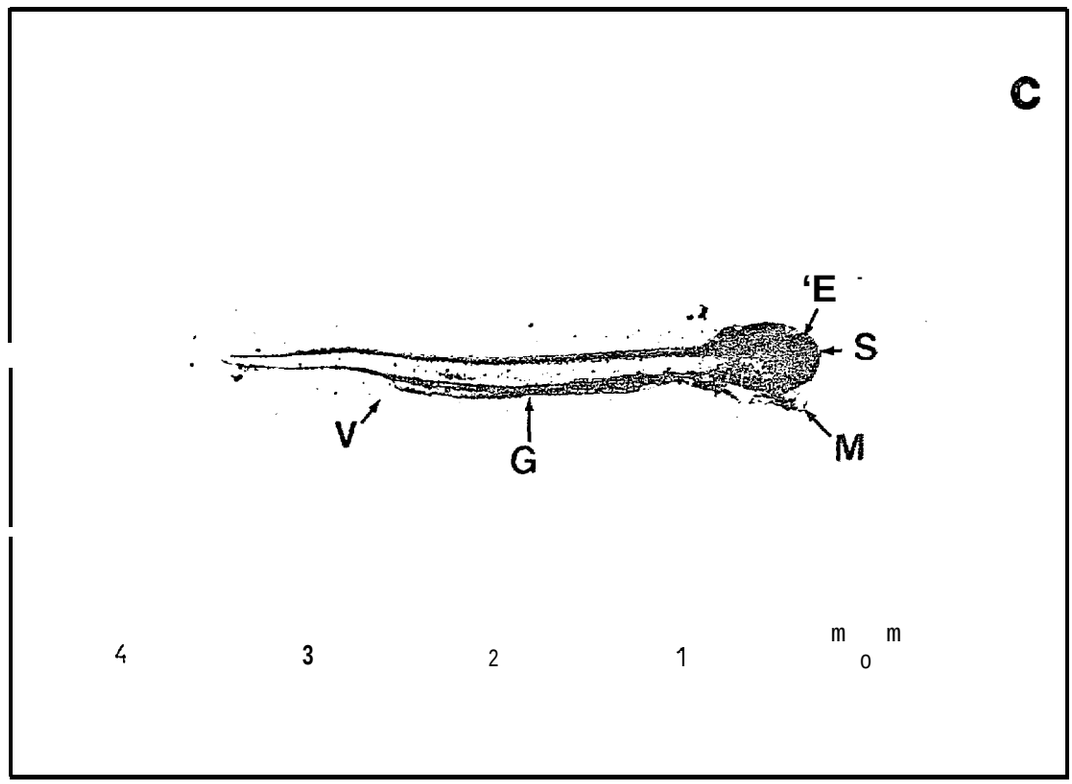
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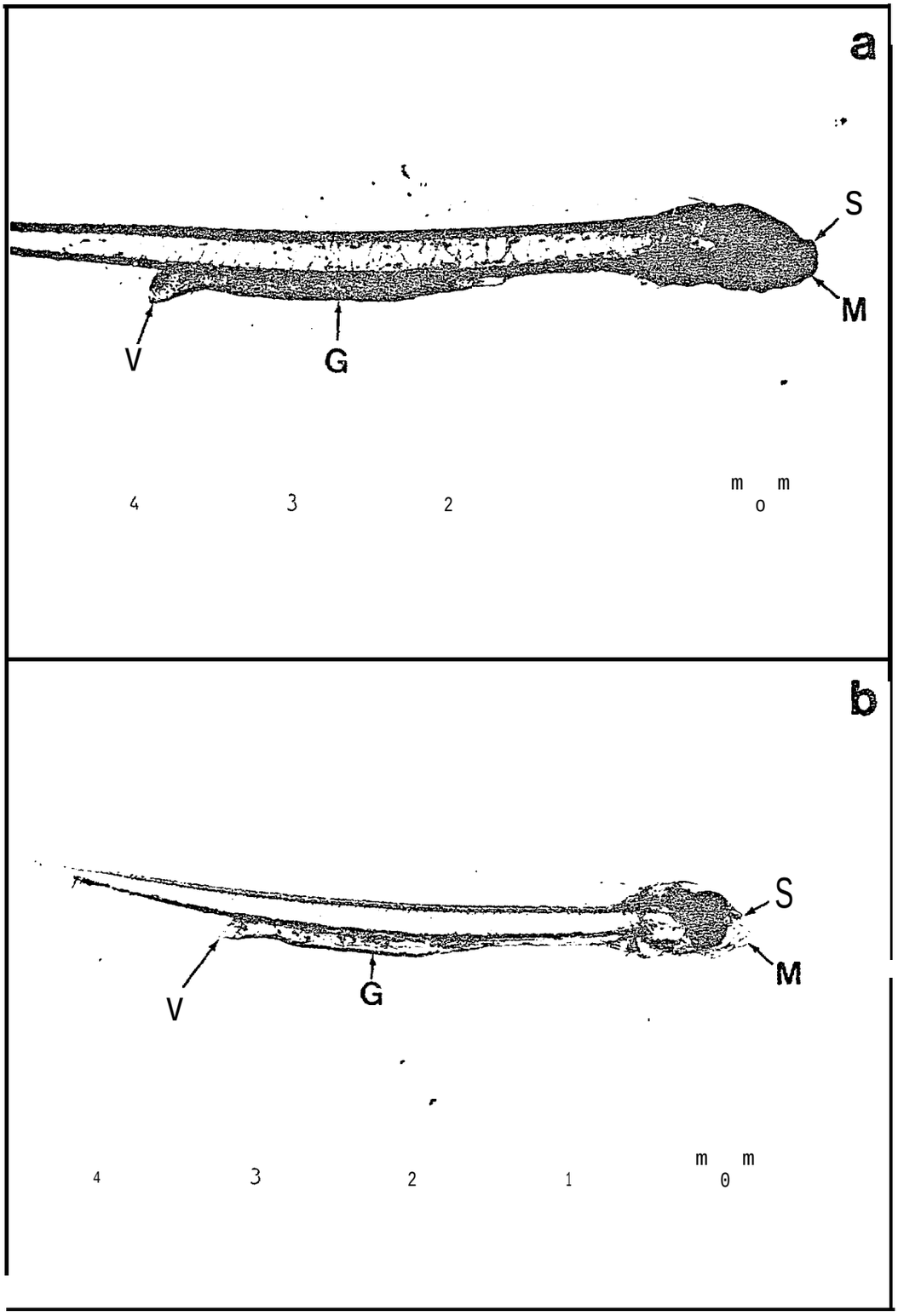
Plate II-11(a-b). Representative photomicrographs of whole-mounted larval northern anchovy from the control (a) and low (b) concentration exposure regimes following 14 days of exposure. Note the shorter head, smaller eye, the better developed vent, and the more blunted mandible in the low concentration larvae. Structures labeled as follows: Snout (S), mouth (M), gut (G), and vent (V).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behavior of the larvae, since those in the control and low concentration remained near the G. splendens patch, while larvae in the medium concentration retained their head-down floating posture.

Three replicate culture aquaria were terminated from each of the remaining test concentrations on day 6. All larvae present in the medium concentration possessed poorly developed jaws and lacked food in their gut. All larvae in the control and low concentration regimes possessed normally developed jaws and most contained food in their gut (Table 11-59).

On day 7, larvae were spread throughout the water column in the control and low concentration regimes. Larvae were observed to actively move about the aquaria, alternating between swimming and "striking" activities. No larvae were observed in the medium concentration regime.

Larval behavior did not change noticeably throughout the remainder of the experimental period. The remaining control and low concentration culture aquaria were terminated on day 14 due to absence of visual larval sightings in two of the three low concentration aquaria.

c. Adult Experiments

1) Hydrocarbon Exposure. Adult northern anchovy (Engraulis mordax) were exposed to control seawater and three WSF test solution concentrations for approximately 16.5 weeks. Table II-60 summarizes the hydrocarbon concentration levels for each of the aqueous exposure regimes. As Table II-60 indicates, the control seawater contained relatively high background hydrocarbons levels largely attributable to the presence of diethyltetrahydrofuran. Excluding the diethyltetrahydrofuran, the mean hydrocarbon level in the control seawater was 2.8 µg/l. Similarly corrected values for the low, medium and high exposure level test solutions were 5.8, 24.8, and 177 µg/l, respectively. Statistical evaluation (Student's t-test) of the background-corrected total hydrocarbon concentration levels revealed that hydrocarbon concentrations were significantly different ($p < 0.05$) at each of the four exposure levels.

Table II-59. Larval anchovy gut examination for food content.

Concentration	Mean Number of Surviving Larvae	Mean Number of Surviving Larvae With Food in Gut	Mean Percent Surviving Larvae With Food in Gut
Control	26.3	24.6	93.6
Low	14.6	13.3	90.0
Medium	10.6	0.0	0.0

Table II-60. Hydrocarbon exposure concentrations for adult northern anchovy experiments.

Exposure Level	Hydrocarbon Concentration (µg/l)[a] Volatile Fraction	Extractable Fraction[b]	Total[c]
Control	0.3±0.2	2.5±1.8 [7.3±4.6]	2.8±1.8 [7.6±4.6]
Low	2.2±2.0	3.6±3.7 [8.5±6.1]	5.8±3.9 [10.7±6.2]
Medium	20.0±13.1	4.8±2.3 [9.8±6.2]	24.8±10.2 [29.8±11.6]
High	156±68	21.2±23.3 [28.5±22.5]	177±58 [185±56]

a Data are reported as the mean ±1 standard deviation.

b Extractable hydrocarbon values are reported corrected for the presence of diethyl tetrahydrofuran and uncorrected values are also presented in brackets.

c Total hydrocarbon values represent the sum of the average daily volatile fraction plus the weekly extractable fraction. Values are also reported both corrected and uncorrected for diethyltetra hydrofuran.

2) Hydrocarbon Tissue Burden. Over the course of the 120-day exposure period, northern anchovy were randomly selected for sacrifice and subsequent hydrocarbon tissue burden determination at 30-day intervals. fish from control, low, and medium concentrations were sacrificed at 30, 60, 90 and 120

days. Due to excessive mortality, however, the high concentration test was terminated after 30 days of exposure. As a result, tissue burden characterizations were only performed once on organisms from the high exposure level. At each of the four intervals organisms were sacrificed, dissected, and selected tissues were removed for hydrocarbon analysis. This design enabled examination of bioaccumulation phenomena over discrete exposure intervals, rather than a single evaluation of bioaccumulation at the termination of the exposure period.

Selected tissues included muscle and a combined head/viscera sample. The head/viscera sample included the entire skull and its contents, brain, spinal cord, and all of the visceral organs. Biomass limitations precluded the use of individual organs for petrogenic hydrocarbon characterizations. All samples represented composites of tissues from a number of individual organisms. Triplicate composites were analyzed on all occasions. Additionally, triplicate analyses were performed on both tissues from background organisms.

Petrogenic hydrocarbon tissue burden data are presented as tabular summaries, and detailed characterization data are presented in the data appendix. Hydrocarbon data are grouped into five categories: benzenes, indenes, naphthalenes, thiophenes and furans, and phenanthrenes. Each category is composed of the parent compound(s) and its alkyl-substituted homologs. The total petrogenic hydrocarbon tissue burden is also given in each table, and represents the total of the five categories plus any additional petroleum derived hydrocarbons which were identified.

Results of muscle tissue burden characterizations from organisms sacrificed at day 30 and day 60 are presented in Tables 11-61 and II-62, respectively. As Table II-61 indicates, petrogenic hydrocarbons were not detected in background samples or in muscle tissue samples from control, low or medium concentrations. However, within the 30-day exposure period significant bioaccumulation of petroleum derived hydrocarbons had occurred in muscle tissue from anchovy exposed to the high concentration. The mean tissue burden was 1653 rig/g; which was composed largely of benzene and naphthalene derivatives. Additionally, substituted indenes and thiophenes were identified in these tissue extracts at significant concentration levels. A total of 19 individual petrogenic hydrocarbons were identified at mean concentrations as high as 358 ng/g.

Table II-62 details the muscle tissue burden data from fish exposed to WSF test solutions for 60 days. Although no petrogenic hydrocarbons were detected in muscle tissue from control and low concentration fish, bioaccumulation had occurred at the medium concentration after 60 days. As Table II-62 indicates, the mean tissue burden for fish from the medium concentration was 271 rig/g. Alkyl-substituted naphthalenes were the predominant species identified, although benzenes and indenes were also present at significant levels. A relatively diverse array of petrogenic hydrocarbons were identified, including seven benzenes, six naphthalenes, four indenes, and one thiophene.

Results of muscle tissue burden characterizations from E. mordax sacrificed at day 90 and day 120 are presented in Tables II-63 and II-64, respectively. As evidenced by Table II-63, no petrogenic hydrocarbons were detected in muscle tissue extracts from control fish. However, after 90 days of exposure, petrogenic hydrocarbons were observed to bioaccumulate in muscle tissues from low concentration organisms and mean tissue levels had

Table II-61. Petrogenic hydrocarbon burden in adult northern anchovy muscle tissue following 30 days 01 exposure.

Toxicant Category[b]	Exposure Level[a] Bkg[c]	Control	LOW	Medium	High
Benzenes	ND	ND	ND	ND	730±500
Indenes	ND	ND	ND	NO	142±124
Naphtha lenes	ND	ND	No	ND	721:437
Thiophenes and Furans	NO	NO	No	NO	60±27
Phenanthrenes	ND	ND	ND	NO	ND
Total Petrogenic Hydrocarbons Identified	NO	ND	ND	NO	1653±972

NO = None detected.

a Concentration data reported as mean ±1 standard deviation in ng/g dry weight.

b Each categor-ys composed of the parent compound and alkyl -substituted homologs.

c Bkg = Background tissue sample {tissue sample from organi sms collected at the same time as those used fcr exposure studies).

Table II-62. Petrogenic hydrocarbon burden in adult northern anchovy muscle tissue following 60 days 01 exposure.

Toxicant Category[b]	Exposure Levels Bkg[c]	Control	Low	Medium	Hi gh[d]
Benzenes	ND	ND	NO	66±58	
Indenes	ND	NO	ND	42±51	
Naphthalenes	No	NO	ND	151±135	
Thiophenes and Furans	N D	N O	No	12±4	
Phenanthrenes	ND	ND	NO	ND	
Total Petrogenic Hydrocarbons Identified	NO	No	ND	271±237	

NO = None detected.

a Concentration data reported as mean ±1 standard deviation in ng/g dry weight.

b Each categoryis composedof the parentcompoundand alkyl-substituted homologs.

c Bkg = Background tissue sample (tissue sample from organisms collected at the same time as those used for exposure studies).

d High exposure level study previously terminated.

increased in organisms from the medium concentration. The mean petrogenic hydrocarbon tissue burden in muscle samples from low concentration fish was 111 rig/g, which was composed of alkyl-substituted benzenes, indenes, naphthalenes and thiophenes. Although the naphthalenes dominated the cumulative tissue burden level, 27 different petrogenic hydrocarbons were identified.

Similarly, muscle tissue extracts from organisms exposed to the medium concentration contained a diverse complement of petrogenic hydrocarbons. Parent and alkyl-substituted benzenes, indenes, naphthalenes and thiophenes were identified at individual concentration levels of 2 to 88 rig/g. A total of 39 individual petrogenic compounds were detected at a mean cumulative concentration level of 377 rig/q. Naphthalene derivatives were again observed to be the predominant compounds, with benzenes and indenes also present at significant levels. Although the mean tissue burden of organisms exposed to the medium concentration for 90 days was greater than that observed after 60 days, the tissue burdens were not statistically different (Student's t-test).

Table II-63. Petrogenic hydrocarbon burden in adult northern anchovy muscle tissue following 90 days of exposure.

Toxicant Category[b]	Exposure Levels Bkg[c]	Control	Low	Medium	High[d]
Benzenes	No	ND	10±17	99±86	
Indenes	NO	ND	7±13	51±45	
Naphthalenes	NO	ND	91±72	225~200	
Thiophenes and Furans	NO	No	*	2 ±3	
Phenanthrenes	N O	ND	ND	ND	
Total Petrogenic Hydrocarbons Identified	NO	NO	111±100	377±327	

ND = None detected * = Trace amount detected

a Concentration data reported as mean ±1 standard deviation in rig/g dry weight.

b Each category is composed of the parent compound and alkyl-substituted homologs.

c Bkg = Background tissue sample [tissue sample from organisms collected at the same time as those used for exposure studies).

d High exposure level study previously terminated.

Table II-64. Petrogenic hydrocarbon burden in adult northern anchovy muscle tissue following 120 days of exposure.

Toxicant Category[b]	Exposure Levels Bkg[c]	Control	Low	Medium	High[d]
Benzenes	ND	ND	86±70	107±16	
Indenes	NO	NO	16±13	95±45	
Naphtha lenes	ND	No	91±78	299±214	
Thiophenes and Furans	ND	NO	6±1	4±4	
Phenanthrenes	No	ND	ND	ND	
Total Petrogenic Hydrocarbons Identified	NO	NO	199±163	505±274	

NO = None detected

a Concentration data reported as mean ±1 standard deviation in rig/g dry weight.

b Each category is composed of the parent compound and alkyl-substituted homologs.

c Bkg = Background tissue sample (tissue sample from' organ isms collected at the same time as those used for exposure studies).

d High exposure level study previously terminated.

Petrogenic hydrocarbon levels in muscle tissues from organisms sacrificed at the end of the experiment are presented in Table 11-64. The mean total petrogenic hydrocarbon level from organisms exposed to the low concentration was 199 rig/g, roughly twice the level observed after the 90 day exposure period. The tissue burden was composed predominately of alkyl-substituted benzenes and naphthalenes, with considerably lower levels of indenes and thiophenes. A total of 16 different petrogenic hydrocarbons were identified, with individual concentrations levels from 3 to 35 rig/g. Due to the variability among individual data points, the mean total tissue burden after 120 days of exposure was not found to be significantly different ($p > 0.05$) than that observed for the organisms exposed for 90 days.

Muscle extracts from anchovy exposed to the medium concentration contained a mean total petrogenic hydrocarbon content of 505 rig/g. Of this total, approximately 60% was contributed by naphthalene derivatives, 21% by benzene derivatives and 19% from alkyl-substituted indenes. Concentrations of individual petrogenic hydrocarbons ranged from 2 to 109 rig/g and 27 different species were identified. Again due to the variability of individual samples, no statistically significant difference ($p > 0.05$) was observed between muscle tissue burdens at 120 days of exposure and those at 60 or 90 days of exposure.

Figure 11-21 provides a graphical summary of the muscle tissue burden data for adult northern anchovy. This graph depicts the total petrogenic hydrocarbon levels in muscle tissues from organisms exposed to each of the four exposure regimes. Data are included for exposure periods of 30, 60, 90 and 120 days. Although the high concentration regime was terminated after 30 days, tissue burden data from low and medium concentration fish indicated a progressive bioaccumulation with increasing exposure duration.

Bioaccumulation of petrogenic hydrocarbons in head/visceral tissues followed a pattern similar to that observed in muscle tissues. Results of tissue burden characterizations from northern anchovy sacrificed at day 30 and day 60 are presented in Tables II-65 and II-66, respectively. As evidenced by Table II-65, no bioaccumulation of petrogenic hydrocarbons was detected in background samples or in tissue extracts from control, low and medium concentration. During the 30-day exposure period organisms exposed to the high concentration did exhibit significant bioaccumulation in head/visceral tissues. The mean petrogenic hydrocarbon tissue burden was 6701 rig/g, composed largely of alkyl-substituted benzenes (2514 rig/g) and naphthalenes (2732 rig/g). In addition to these species, substantial levels of substituted indenes were observed (1253 rig/g) and

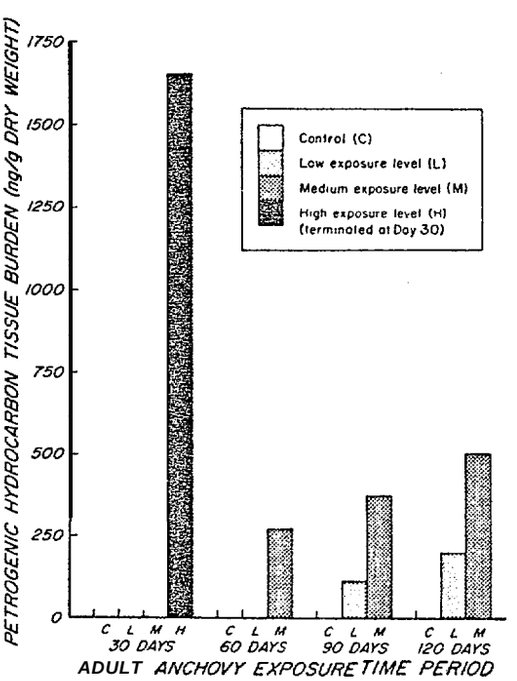


Figure 11-21 . Total petrogenic hydrocarbon burden in adult northern anchovy muscle tissue.

Table II-65. Petrogenic hydrocarbon burden in adult northern anchovy head/viscera tissue following 30 days of exposure.

Toxicant Category[b]	Exposure Level[a] Bkg[c]	Control	LOW	Medium	High
Benzenes	ND	ND	ND	ND	2514±1514
Indenes	NO	ND	ND	ND	1253±373
Naphthalenes	ND	ND	ND	ND	2732±958
Thiophenes and Furans	ND	ND	ND	ND	202±69
Phenanthrenes	ND	ND	ND	No	ND
Total Petrogenic Hydrocarbons Identified	ND	ND	ND	ND	6701±2744

NO = None detected.

a Concentration data reported as mean ±1 standard deviation in ng/g dry weight.

b Each category is composed of the parent compound and alkyl-substituted homol ogs.

[c] Bkg = Background tissue sample (tissue sample from organisms collected at the same time as those used for exposure studies).

Table II-66. Petrogenic hydrocarbon burden in adult northern anchovy head/viscera tissue following 60 days of exposure.

Toxicant Category[b]	Exposure Level[a] Bkg[c]	Control	Low	Medium	High[d]
Benzenes	ND	ND	5±8	42±47	
Indenes	ND	ND	5±9	21±18	
Naphthalenes	ND	ND	41±66	235±189	
Thiophenes and Furans	ND	ND	ND	ND	
Phenanthrenes	ND	ND	ND	ND	
Total Petrogenic Hydrocarbons Identified	ND	NO	51±70	298±251	

ND = None detected.

[a] Concentration data reported as mean ±1 standard deviation in rig/g dry weight.

b Each category is composed of the parent compound and alkyl-substituted hmologs.

c Bkg = Background tissue sample (tissue sample from organisms collected at the same time as those used for exposure studies).

d High exposure level study previously terminated.

several thiophene derivatives were detected. A total of 35 individual petrogenic hydrocarbons were identified at mean concentration levels in excess of 1100 rig/g.

Table II-66 details head/viscera petrogenic hydrocarbon tissue burden data from northern anchovy exposed to the WSF test solutions for 60 days. Petrogenic hydrocarbons were not detected in tissue extracts from control organisms. However, at the end of the 60 day exposure period bioaccumulation had occurred in both the low and medium concentrations. Head/visceral tissue extracts from anchovy exposed to the low concentration exhibited a mean petrogenic hydrocarbon tissue burden of 51 rig/g. Roughly 80% of this total was attributable to alkyl-substituted naphthalenes, while the remaining 20% was composed of indene and benzene derivatives.

The bioaccumulation pattern in the medium concentration was very similar. The mean total tissue burden in head/visceral samples from fish exposed to the medium concentration for 60 days was 298 ng/g. As was the case for the low concentration, roughly 79% of the total was due to naphthalene derivatives, and approximately 21% was attributable to substituted benzenes and indenes. The concentration of individual components ranged from 6 to 140 rig/g, and a total of 8 petrogenic hydrocarbons were identified.

Tables II-67 and II-68 present hydrocarbon tissue burden data for head/visceral tissues exposed to WSF test solutions for 90 and 120 days, respectively. As evidenced by Table II-67, no bioaccumulation of petrogenic hydrocarbons occurred in control organisms; however bioaccumulation in WSF-exposed fish intensified somewhat between days 60 and day 90. After a 90-day exposure period, head/viscera from fish in the low concentration exhibited a mean tissue burden of 102 rig/g. The dominant compounds were alkyl-substituted naphthalenes with lesser amounts of benzene and indene derivatives. Nine distinct petrogenic hydrocarbons were identified at individual concentration levels of 2 to 58 rig/g. Statistical analysis of these data showed no significant difference {p >0.05) between tissue burdens of organisms' exposed for 60days and these exposed for 90 days.

Adult E. mordax exposed to the medium concentration for 90 days exhibited a mean petrogenic hydrocarbon content in head/visceral tissues

of 298 rig/g. This total was comprised of approximately 49% alkyl-substituted naphthalenes, 32% substituted indenes, and 19% benzene derivatives. Within these three categories, a diverse complement of individual hydrocarbons were identified, including 14 benzenes, 11 indenes and eight naphthalenes. Individual concentration levels ranged from 2 to 110 rig/g. No statistically significant difference ($p > 0.05$) was observed between mean tissue burden levels at day 60and day 90.

Table II-68 summarizes petrogenic hydrocarbon tissue burden data for head/visceral tissue from E. mordax exposed to WSF test solutions for 120 days. Petrogenic hydrocarbons were not detected in any of the control organisms. The mean total tissue burden for organisms exposed to the low concentration was determined to be 229 ng/g. The petrogenic hydrocarbon content was composed predominately of alkyl-substituted benzenes and naphthalenes (approximately 45% each) with the remaining 10% attributable to indene and thiophene derivatives. A total of 24 specific petrogenic hydrocarbons were identified in these tissue extracts, including 15 alkyl-benzenes, at individual concentration levels of 2 to 38 rig/g. Statistical analysis of these data (Student's t-test) showed mean total tissue burdens at day 120 to be significantly different from those at day 60 ($p < 0.05$), but not significantly different than those at day 90 ($p > 0.05$).

Head/visceral tissue samples from organisms exposed to the medium concentration for 120 days exhibited a mean tissue burden of 606 rig/g. Alkyl-substituted naphthalenes were the predominant components (48%), although significant levels of benzene derivatives (30%) and substituted indenes (22%) were also identified. A total of 42 distinct petrogenic hydrocarbons were identified, including 16 benzenes, 13 indenes, 11 naphthalenes and 2 thiophenes. Compound concentration levels ranged from 4 to 153 ng/g. Statistical analysis of these data (Student's t-test) revealed that the mean tissue burden observed after 120 days of exposure was significantly different ($p < 0.05$) from that determined following 60 and 90 days of exposure.

Figure II-22 illustrates the total head/viscera tissue burdens from organisms exposed to the control, low, medium, and high concentration regimes. In addition, data are presented for each exposure concentration at the 30, 60,

Table II-67. Petrogenic hydrocarbon burden in adult northern anchovy head/viscera tissue following 90 days of exposure.

Toxicant Category[b]	Exposure Level[a] Bkg[c]	Control	Low	Medium	High[d]
Benzenes	ND	ND	21±14	58±52	
Indenes	ND	ND	14±24	94±58	
Naphthalenes	ND	ND	67±101	146±119	
Thiophenes and Furans	ND	ND	.	ND	
Phenanthrenes	ND	ND	ND	ND	
Total Petrogenic Hydrocarbons Identified	ND	ND	102±137	298±226	

ND = None detected . = Trace amount detected

[a] Concentration data reported as mean ±1 standard deviation in ng/g dry weight.

[b] Each category is composed of the parent compound and alkyl-substituted homologs.

[c] Bkg = Background tissue sample (tissue sample from organisms collected at the same time as those Uses for exposure studies).

[d] High exposure level study previously terminated.

Table ii-68. Petrogenic hydrocarbon burden in adult northern anchovy head/viscera tissue following 120 days of exposure.

Toxicant Category[b]	Exposure Level[a] Bkg[c]	Control	Low	Medium	High[d]
Benzenes	ND	ND	101±64	184±109	
Indenes	ND	ND	15±8	131±62	
Naphthalenes	ND	ND	106±46	291±94	
Thiophenes and Furans	ND	ND	7±6	*	
Phenanthrenes	ND	ND	ND	ND	
Total Petrogenic Hydrocarbons identified	ND	ND	229±123	606±251	

ND = None detected * = Trace amount detected

[a] Concentration data reported as mean ±1 standard deviation in ng/g dry weight.

[b] Each category is composed of the parent compound and alkyl-substituted homologs.

[c] Bkg . Background tissue sample (tissue sample from organisms collected at the same time as those used for exposure studies).

[d] High exposure level study previously terminated.

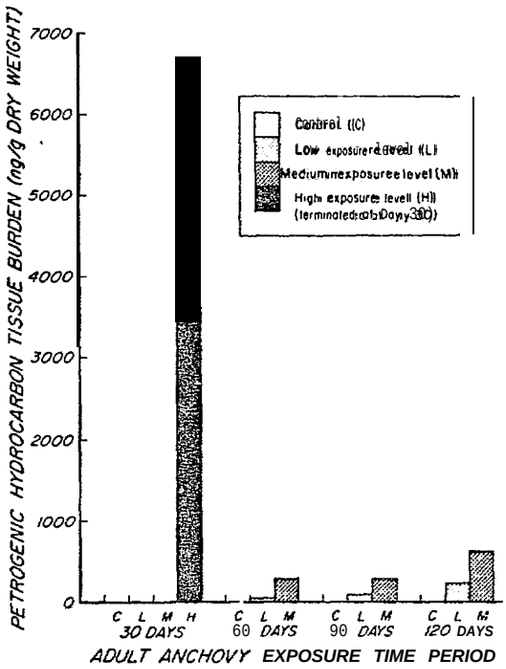


Figure 11-22 . Total petrogenic hydrocarbon burden in adult northern anchovy head/ viscera tissue.

Table II-69. Petrogenic hydrocarbon burden (rig/g) exposure summary of adult northern anchovy muscle and head/viscera **tissues.**

Exposure Condition	Petrogenic Hydrocarbon Level [a] Muscle	Head/Viscera
30 Day		
Control	NO	ND
Low	ND	ND
Medium	ND	ND
High	1653±972	6701±2744
60 Day		
Control	No	ND
Low	ND	51+70
Medi urn	271+237	298±251
90 Day		
Control	ND	ND
Low	111+100	102+137
Medium	31 7+327	298±226
120 Day		
Cent rol	NO	ND
Low	199+163	229+123
Medium	505:274	606± 251

ND = None detec ted

a Total tissue burden of hydrocarbons attributable to petroleum origin; data are means +1 standard deviation.

90 and 120 day exposure intervals. The high concentration was terminated at day 30, thus tissue burdens at days 60, 90 and 120 are only presented for control, low and medium concentration fish, As this figure indicates, continued exposure to the WSF resulted in a progressive bioaccumulation of petrogenic hydrocarbons.

A comparison of petrogenic hydrocarbon tissue burden data for the two anchovy target tissues is presented in Table II-69. These data are. presented as total tissue burdens, representing the summation of all compounds which could be directly linked to the parental crude oil toxicant and distinguished from hydrocarbons of biogenic origin. Data are presented for target tissues from each of the exposure regimes, and at each of the four exposure intervals. As evidenced by Table II-69, the overall bioaccumulation pattern was very similar in both target tissues. The only pronounced differences observed were the greater bioaccumulation in head/visceral tissues from high concentration fish at day 30, and the more rapid bioaccumulation in head/visceral tissues from organisms exposed to the low concentration. At all other exposure levels and exposure intervals no statistically significant difference ($p > 0.05$) was observed between bioaccumulation of petrogenic hydrocarbons in muscle tissue and that in head/visceral tissue extracts.

Accurate calculation of bioaccumulation factors was difficult in the adult E. mordax study because petrogenic hydrocarbons of interest were not detected with sufficient frequency to determine mean WSF concentration levels over the course of the experiment. Petrogenic hydrocarbons identified in the parental crude and in exposed tissue extracts were generally present in the WSF test solutions at concentrations

below the detection limit of approximately 0.1 µg/l. For these reasons, . . bioaccumulation factors are only estimated for adult anchovy target tissues.

Table II-70 presents petrogenic hydrocarbon tissue levels and estimated bioaccumulation factors for individual compounds which were not routinely detected in the WSF test solutions. The data in this table are categorized in the same manner as that used for presentation of the adult anchovy tissue burden data. Each category is composed of the parent hydrocarbon and its alkyl-substituted homologs. Petrogenic hydrocarbon tissue burden levels are presented as concentration ranges for the individual compounds identified in each category. The estimated bioaccumulation factors were calculated using the assumption that the mean aqueous exposure level was 0.1 µg/l for each of the individual hydrocarbons. The 0.1 µg/l aqueous exposure level is derived from analytical detection limit considerations for aromatic hydrocarbons of the type identified in adult anchovy tissue extracts.

Estimated bioaccumulation factors for head/viscera tissue from fish exposed to the high concentration for 30 days were as great as 2720 for alkyl-substituted benzenes, 3250 for indene derivatives, and 11030 for substituted naphthalenes. Estimated bioaccumulation factors for muscle tissue from fish similarly exposed were as high as 1120 for benzenes, 780 for indenes and 3580 for alkyl naphthalenes.

Maximum estimated bioaccumulation factors for benzenes, indenes and naphthalenes in head/viscera tissue from adult anchovy exposed to the

Table II-70. Estimated bioaccumulation factor ranges for petrogenic hydrocarbons in adult northern anchovy head/viscera and muscle tissues.

		Benzenes		Indenes		Naphthalenes		Thiophenes/ Furans	
Tissue	Exposure Condition	Tissue Burden[a]	BF[b]	Tissue Burden[a]	BF[b]	Tissue Burdens	BF[b]	Tissue Burden[a]	BF[b]
Head/Viscera	30 Day Exposure High Level	9-272	90-2720	107-325	1070-3250	82-1103	820-11030	202	2020
	60 Oay Exposure Low Level	5	50	5	50	16-25	160-250	NO	
	Medium Level	6-22	60-220	21	210	15-140	150-1400	NO	
	90 Oay Exposure Low Level	21	210	2-10	20-100	3-58	30-580	NO	
	Medium Level	2-18	20-180	2-22	20-220	2-110	20-1100	NO	
	120 Oay Exposure Low Level	3-32	30-320	4-11	40-110	2-38	20-380	7	70
	Medium Level	5-49	50-490	4-46	40-460	12-153	120-1530	ND	·
Muscle	30 Day Exposure High Level	42-112	420-1120	6-78	60-780	41-358	41 0-35LSO	60	600
	60 Day Exposure Medium Level	4-17	40-170	10-17	100-170	9-82	90-820	12	120
	90 Oay Exposure Low Level	2-3	20-30	2-3	20-30	2-40	20-400	NO	
	Medium Level	4-23	40-230	2-18	20-180	2-88	20-880	2	20
	120 Day Exposure Low Level	3-25	30-250	6-8	60-80	5-3s	50-350	6	60
	Medium Level	7-42	70-420	2-22	20-220	2-109	20-1090	4	40

NO = None detected.

a Data represent the range of mean Content.rati ons (ng/g dry weight] for individual hydrocarbons identified in tissue extracts from organisms exposed to the specified conditions.

b BF . estimated bioaccumulation factor ranges, assuming a mean aqueous exposure level of 0.1 µg/l for individual hydrocarbons in each category.

medium concentration for 60 days were 220, 210, and 1400, respectively. Corresponding data for muscle tissues were determined to be 170 for both benzenes and indenes, and 820 for substituted naphthalenes. After 90 days of exposure at the medium concentration, maximum estimated bioaccumulation factors for both head/viscera and muscle tissues were very similar to those observed at day 60. At termination of the adult anchovy study maximum estimated bioaccumulation factors in head/viscera tissues were 490 for alkyl benzenes, 460 for indene derivatives, and 1530 for substituted naphthalenes. Corresponding data for similarly exposed anchovy muscle tissues were 420 for benzenes, 220 for indenes and 1090 for substituted naphthalenes.

As expected, estimated bioaccumulation factors were generally lower in tissues from organisms exposed to the low concentration. After 60 days of exposure to the low concentration, bioaccumulation was not detected in anchovy muscle tissues. However, head/viscera tissue from similarly exposed fish exhibited estimated bioaccumulation factors ranging from 50 to 250. After 90 days of exposure to the low concentration, bioaccumulation had occurred in both target tissues. Maximum estimated bioaccumulation factors for alkyl-substituted benzenes, indenes and naphthalenes in head/viscera tissue were calculated to be 210, 100, and 580, respectively. Corresponding data for muscle tissue were calculated to be 30 for both benzenes and indenes, and 400 for alkyl naphthalenes. Maximum estimated bioaccumulation factors for fish exposed for 120 days were similar to those observed after 90 days of exposure. The only significant exception was an increase in the maximum estimated bioaccumulation factor for benzenes in muscle tissue from 30 at day 90to 250 at day 120.

In conclusion, several general observations regarding bioaccumulation of petrogenic hydrocarbons in adult anchovy target tissues are noteworthy. In general, the bioaccumulation patterns at each exposure concentration and at each exposure interval were very similar for both tissues. This was true both in terms of the pattern itself and the absolute levels of bioaccumulation. Similarly, at all exposure regimes and intervals, alkyl-substituted naphthalenes were bioaccumulated to the greatest degree and thiophenes to the least. Bioaccumulation of alkyl-substituted benzenes and indenes occurred at intermediate levels and similar absolute values. Phenanthenes were not observed to bioaccumulate at detectable levels in either of the target tissues.

3) Survivorship. The adult Engraulis mordax experiment was terminated after 120 days of test solution exposure. Following 30, 60, 90, and 120 days of exposure, subsamples of 12 fish were removed from each replicate for determination of hydrocarbon tissue burden and tissue pathology. Since daily activities throughout the entire experiment included the removal of moribund animals, percent survivorship could be determined for these fixed periods of exposure.

Percent survivorship was extremely high in the control, low and medium concentration exposure regimes throughout the entire experiment (Table 11-71). Mean cumulative survivorship in the control, low, and medium concentration regimes ranged between 97.3% and 98.5% after 120 days of exposure (Table II-71). In contrast, cumulative survivorship of test fish in the high concentration exposure regime was very low. Only 50.4% of the test fish in the high concentration survived the initial 30 days of exposure, and all fish had died prior to day 60 of the experiment.

A K-W ANOVA demonstrated that mean cumulative survivors hip after 30 days of exposure was significantly lower in the high concentration than in the control, low or medium concentration regimes (Table II-71). No significant difference in mean cumulative survivorship between the control, low and medium concentration exposure regimes (Table 11-71) was evident after 30, 60, 90, or 120 days of exposure.

4) Growth. Growth of adult E. mordax was assessed by measuring the standard length (SL) and total weight (TW) of individual test fish that were randomly sacrificed from replicates at 30-day intervals during the 120-day experiment. At each sacrificial period (i.e. 30, 60, 90, and 120 days), 12 fish were removed from each replicate within an exposure regime to determine the mean SL and TW of fish in that regime. Prior to initiating the experiment, the mean SL and TW of a representative subsample of the entire test fish population was determined by the random removal and measurement of 50 fish from all replicate tanks combined. Using these initial estimates of mean SL and TW, and the mean SL and TW of fish sacrificed at 30-day intervals, cumulative percentage changes in either parameter could be calculated for fixed exposure periods (i.e. 30, 60, 90, or 120 days).

The mean SL of surviving adult E. mordax followed similar patterns of increase in the control, low, and medium concentration exposure regimes over the 120-day experiment (Table II-72). In general, the mean SL, increased only slightly, ranging from 1.0 to 3.0% in the low and control regimes, respectively. No significant difference in mean SL could be demonstrated between fish from any of these exposure regimes after 30, 60, 90, or 120 days of test solution exposure (Table II-72). Although mortality of fish in the high concentration regime was significantly greater than in the control, low, or medium concentrations, no significant reduction in mean SL was found following 30 days of test solution exposure.

In contrast to mean SL, the mean TW of surviving adult E. mordax increased markedly over the 120 day experiment, with cumulative percentage weight increases ranging from 28.0 to 41.0% in the medium and low concentration regimes, respectively

Table II-71. Mean cumulative percent survivorship of adult Engraulis mordax following 30, 60, 90, and 120 days of test solution exposure. Standard errors are shown in parentheses.

Concentration	0	Exposure Period (Days) 30[a]	60[b]	90[b]	120[b]
Control	100.0	99.6[c] (0.50)	98.8 (0.70)	97.9 (0.80)	97.8 (0.10)
Low	100.0	99.7[c] (0.30)	99.3 (0.40)	98.8 (0.60)	98.5 (0.20)
Medium	100.0	99.9[c] (0.20)	99.4 (0.20)	98.3 (0.30)	97.3 (0.30)
High	100.0	50.4[d] (3.40)	0.0 (-)	-	

[a] Significant difference among groups ($P < 0.05$); K-W ANOVA.

[b] No significant difference among groups ($P > 0.05$); K-W ANOVA.

[c,d] Groups designated by different letters sigrtificantly different ($p < 0.05$) from all other groups: non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

Table II-72. Mean standard length (mm) of adult Engraulis mordax (males and females combined) from pooled replicates following 30,60, 90, and 120 days o! test solution exposure. Standard errors are shown in parentheses.

Concentration 0	Exposure Period (Days) 30a	60[a]	90[a]	120[a]
Control 111.00 (2.3)	117.50 (3.4)	119.60 (2.3)	116.10 (4.3)	120.80 (1.8)
LOW 111.00 (2.3)	118.50 (1.8)	117.90 (2.6)	118.20 (3.2)	119.90 (2'.5)
Medium 111.00 (2.3)	116.30 (2.9)	115.60 (2.3)	116.70 (4.2)	119.30 (3.1)
High 111.00 (2.3)	118.11 (2.4)	-	-	-

[a] Mean standard length not significantly different among groups ($p > 0.05$); K-h' ANOVA.

(Table 11-73). In general, the cumulative percentage weight gain of medium concentration fish was consistently less than that observed for fish from other regimes at all time intervals (Table II-74). There was no significant

Table II-73. Cumulative percent weight gain of adult Engraulis mordax following 30. 60, 90, and 12 0 days of test solution exposure.

Concentration	Exposure Period (Days) 30	60	90	120
Control	18.0	32.0	27.0	38.0
Low	22.0	26.0	33.0	41.0
Medi um	14.0	16.0	23.0	28.0
High	12.0	-		

Table ii-74 . Mean total weight (grams) of adult Engraulis mordax (males and females combined) from pooled replicates following 30,60, 90, and 120 days of test solution exposure. Standard errors are shown in parentheses. All means based on 36 measurements except the high concentration at 30 days (N=24).

Concentration	Exposure Period (Days) 0	30[b]	60[b]	90[a]	120[a]
Control	15.5 (3.5)	18.3[c] (3.7)	10.4[c] (4.5)	19,7 (4.4)	21.4 (5.1)
Low	15.5 (3.5)	18.9[c] (3.9)	19.5[c] (3.9)	20.6 (5.3)	21.9 (5.2)
Medium	15.5 (3.5)	17.6[d] (3.4)	18.0[d] (3.9)	19,1 (4.8)	19.9 (4.5)
High	15.5 (3.5)	17.4[d] (5.1)	-	-	-

[a] Mean total weight of test fish not significantly different ($p > 0.05$); K-W ANOVA,

[b] Mean total weight of test fish significantly different ($p < 0.05$); K-W ANOVA.

[c,d] Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

difference among exposure regimes, however, in the mean TW of surviving fish after 90 or 120 days of exposure, even though the mean TW of medium concentration fish was smaller in both cases.

5) Histopathology. Complete necropsies were Performed on adult northern anchovy, including histopathological study of gill, heart, liver, pancreas, kidney, interrenal gland, stomach, pyloric caecum, intestine, gonad, skeletal muscle, skin, brain, and retina. The only consistent histopathological change observed was evidence of chemical injury to the gills for high concentration fish after 30 days of exposure. This consisted of focal fusion of the tips of secondary lamellae and increased numbers of eosinophilic granular cells at the bases of secondary lamellae (Plates II-12b-c). Additional histopathological effects were not found in anchovy tissue following each exposure period or concentration (Plates 11-12 to II-27).

4. CALIFORNIA MUSSEL (Mytilus californianus)

a. Embryo Experiment

1) Hydrocarbon Exposure. Hydrocarbon exposure data for the California mussel Mytilus californianus embryo experiment are presented in Table II-75. Background hydrocarbon concentration in the control water was approximately 2.8 µg/l. The hydrocarbon concentration in the low level test solution was 6.6 µg/l, while medium and high concentration test solutions were 47.1 and 360.0 µg/l, respectively. As is evidenced by Table II-75, all exposure levels were close to target values.

2) Hydrocarbon Tissue Burden. Mean radioactive tracer uptake by Mytilus californianus embryos was used as an indirect measure of hydrocarbon bioaccumulation. Tracer uptake by embryos was directly proportional to the hydrocarbon concentration in each test solution exposure regime. Tracer levels in embryos ranged from 0.36 to 11.19 cpm/embryo for control and high exposure regimes, respectively (Table 11-76). A K-W ANOVA based on ranked data showed that radioactive tracer uptake was significantly different ($p < 0.05$) among control embryos and those exposed to WSF.

Table II-75. Hydrocarbon exposure summary for California mussel embryo experiment.

Exposure Level	Hydrocarbon Concentration (µg/l)[a] Volatile Fraction	Extractable Fraction	Total
Control	0.9	1.9 [8.4]	2.8 [9.31
Low	5.4	1.2 [6.1]	6.6 [11.51
Medium	41.6	5.5 [14.6]	47.1 [56.2]
High	331	27.9 [311	360 [3621

a Extractable and total hydrocarbon values are reported corrected for the presence of diethyl-tetrahydrofuran and uncorrected values are also presented in brackets.

Table II-76. Mean radioactive tracer (counts per minute/embryo) for M. californianus embryos. Standard error shown in parentheses.

Concentration	Mean cpm/Embryos
Control	0.36 (0.03)[b]
Low	0.62 (0.04)[b]
Medium	3.91 (0.35)[c]
High	11.20 (0.37)[d]

a Tracer uptake significantly different ($p < 0.05$) among groups; K-W ANOVA.

b,c,d Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

An analog to the SNK multiple comparison test was used to determine which specific test solution concentrations resulted in significant bioaccumulation of radioactive tracer in mussel embryos. Application of this test to ranked data revealed that embryos exposed to medium and high concentrations of test solution contained significantly higher ($p < 0.05$) levels of radioactive tracer than control or low concentration animals. Further, those embryos in the high concentration exposure regime bioaccumulated significantly more ($p < 0.05$) tracer than medium exposure animals. There was no significant difference between the tracer levels found in control and low concentration embryos.

3) Survivorship. M. californianus embryos from a stock suspension were aliquoted into each experimental aquarium. Replicate aquaria for control and experimental regimes initially contained 332±17 embryos. Total counts of surviving metamorphosed veligers were made 72 hrs after fertilization and exposure to test solutions (see data appendix).

Survivorship of embryos in control, low, and medium exposure regimes was relatively high compared to that in the high concentration test solution. Mean survivorship ranged from 262 to 303 individuals/replicate for high exposure and control seawater regimes, respectively (Table II-77). A K-W ANOVA indicated that significant differences existed in embryo survivorship in control seawater and test solution exposure regimes. A SNK multiple comparison test revealed that significantly higher mortality ($p < 0.05$) had occurred in the embryos exposed to the high concentration test solution compared to any other exposure regime. Survivorship in the control, low, and medium exposure regimes did not differ significantly.

4) Development. Embryonic M. californianus were monitored during the 72-hr exposure period for delayed metamorphosis and abnormal development. Delayed metamorphosis was indicated by failure of some embryos in each experimental regime to metamorphose from trochophore larvae to veliger stages. Abnormal development was apparent in veligers which had failed to develop a shell or those which developed a malformed shell (see data appendix).

The mean number of mussel trochophores (i.e. indicative of delayed metamorphosis) found in experimental regimes ranged from 2.33 to 11.66 individuals per replicate for medium and high concentrations respectively (Table II-78). A K-W ANOVA on ranked data indicated that there were significant ($p < 0.05$) differences among control and test exposure regimes inthe number of embryos experiencing. delayed metamorphosis. Multiple comparisons using

Plate 11-12 (a-c). Gill of adult northern anchovy from the control group (a) and high (b-c) concentration after 30 days of exposure.

a. Control: Primary lamella extends upward to left. Secondary lamellae (arrows). There is no significant abnormality. X325.

b. High: Note focal fusion of some secondary filaments at top (arrows) cartilage or primary filament (c). X325.

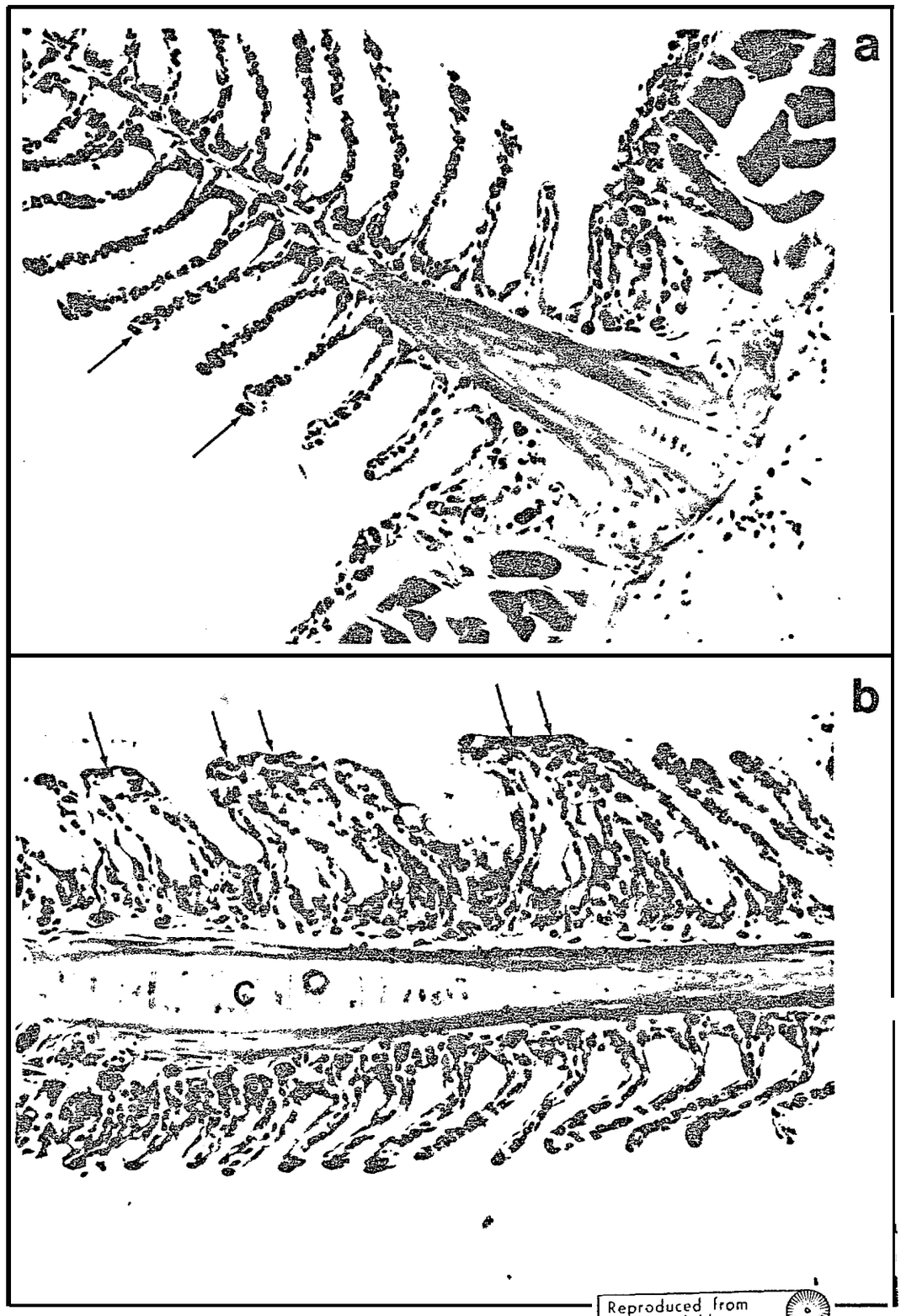


Plate II-12(a-c). (Cent)

c. High: There is increased number of inflammatory cells (I) at base of primary lamellae. X325.

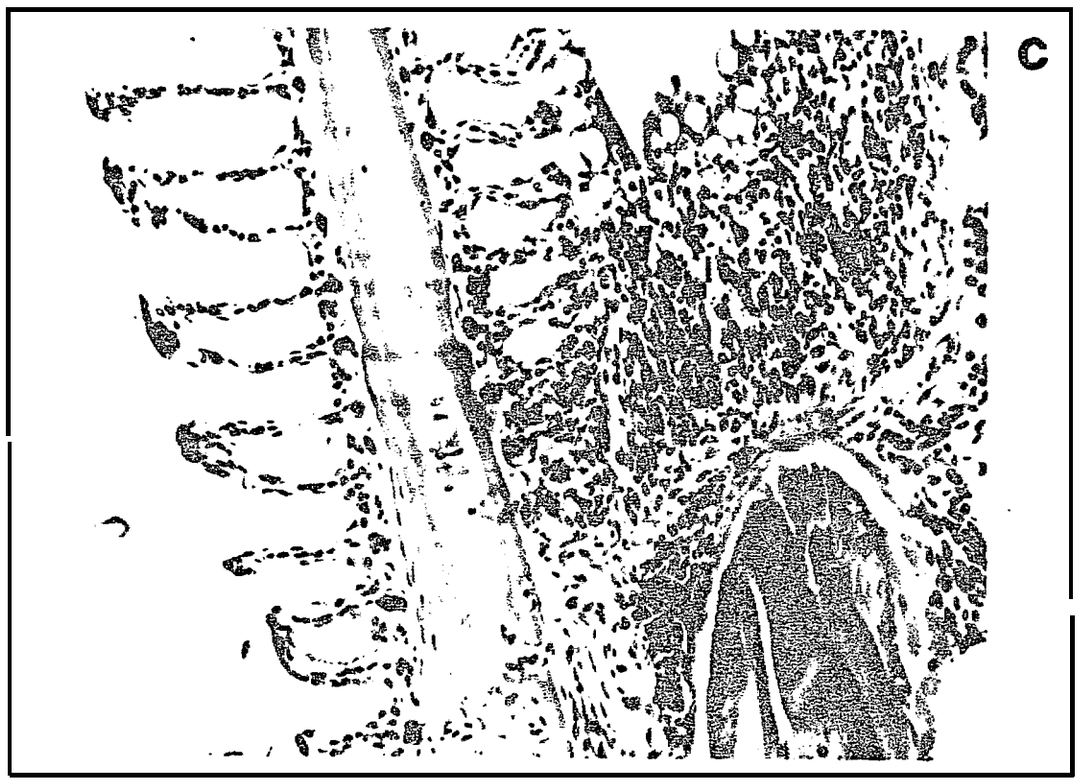

C

Plate II-13 (a-b). Gill of adult northern anchovy from the control group (a) and medium (b) after 60 days of exposure.

a. Gill of adult northern anchovy, control concentration, 60 day exposure. X325.

Il. Gill of adult northern anchovy, medium concentration, 60 day exposure. No significant difference from control. X325.

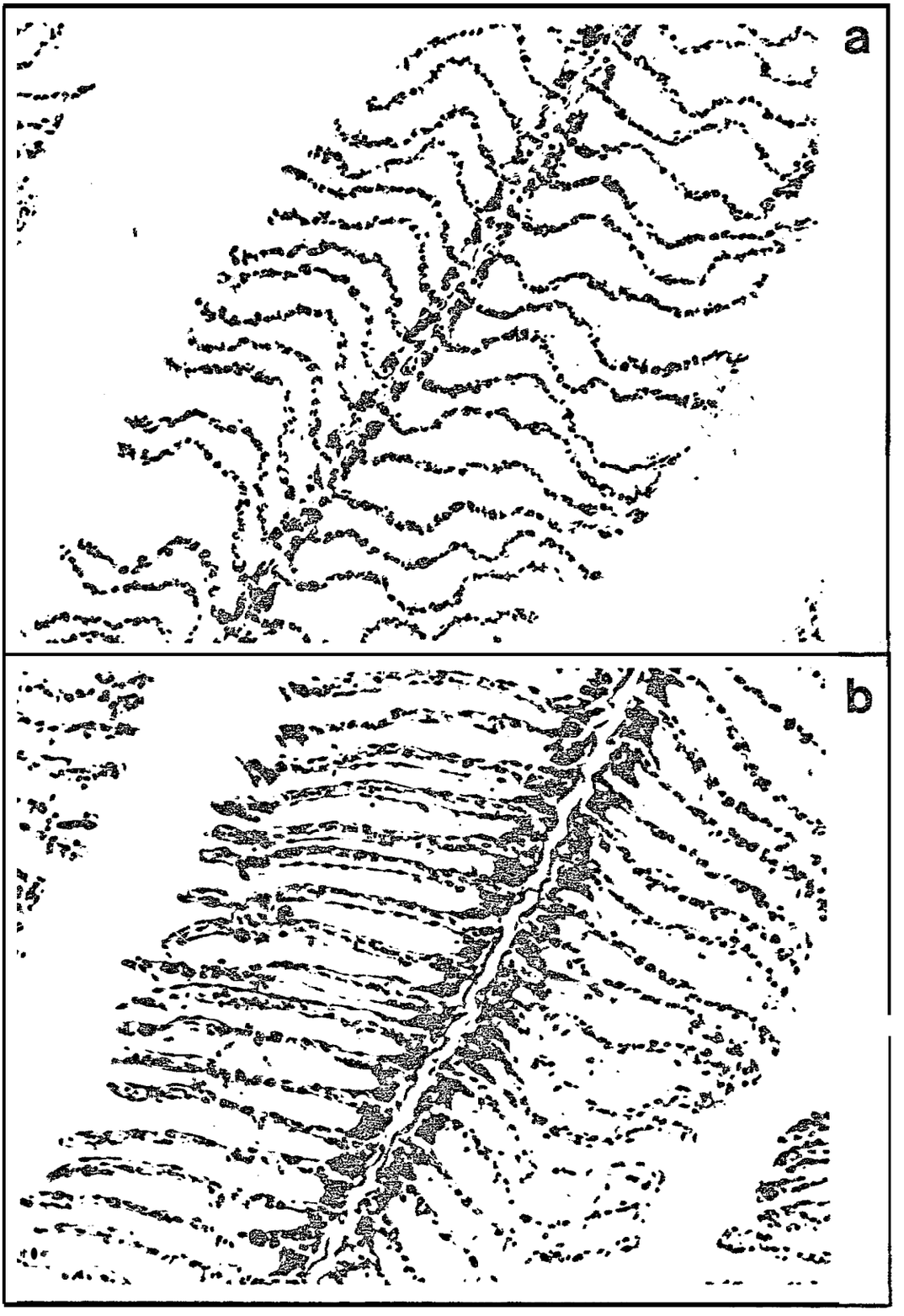

a
b

Plate 11-14 (a-b). Gill of adult northern anchovy from the control group (a) and medium (b) concentration after 90 days of exposure.

a. Gill of adult northern anchovy, control concentration, 90 day exposure. X325.

b. Gill of adult northern anchovy, medium concentration, 90 day exposure. No significant difference from control. X325.

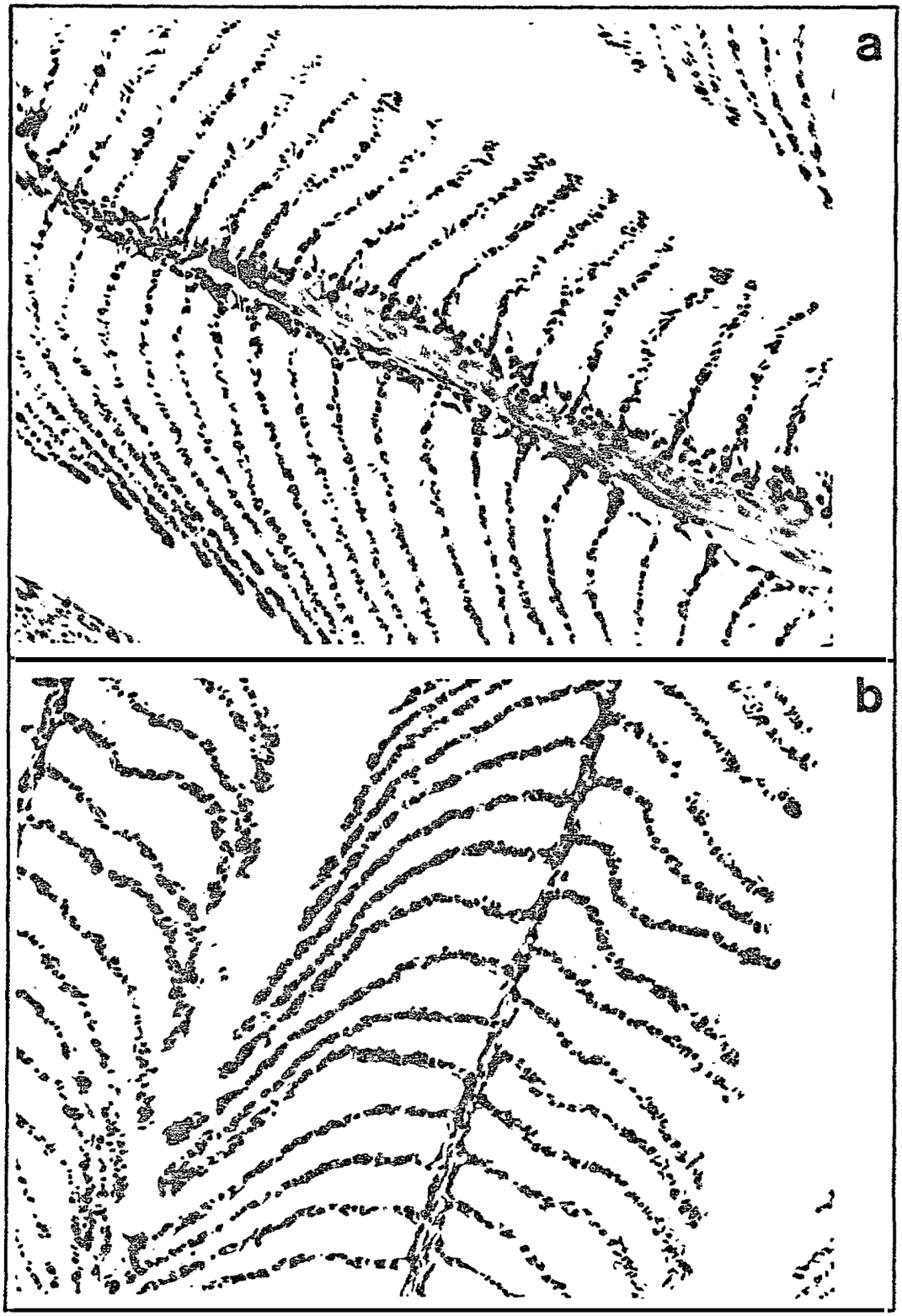

a
b

Plate 11-15 (a-b). Gill of adult northern anchovy from the control group (a) and medium (b) concentration after 120 days of exposure.

a. Gill of adult northern anchovy, control concentration, 120 day exposure. X325.

b. Gill of adult northern anchovy, medium concentration, 120 day exposure. No significant difference from control. X325.

b

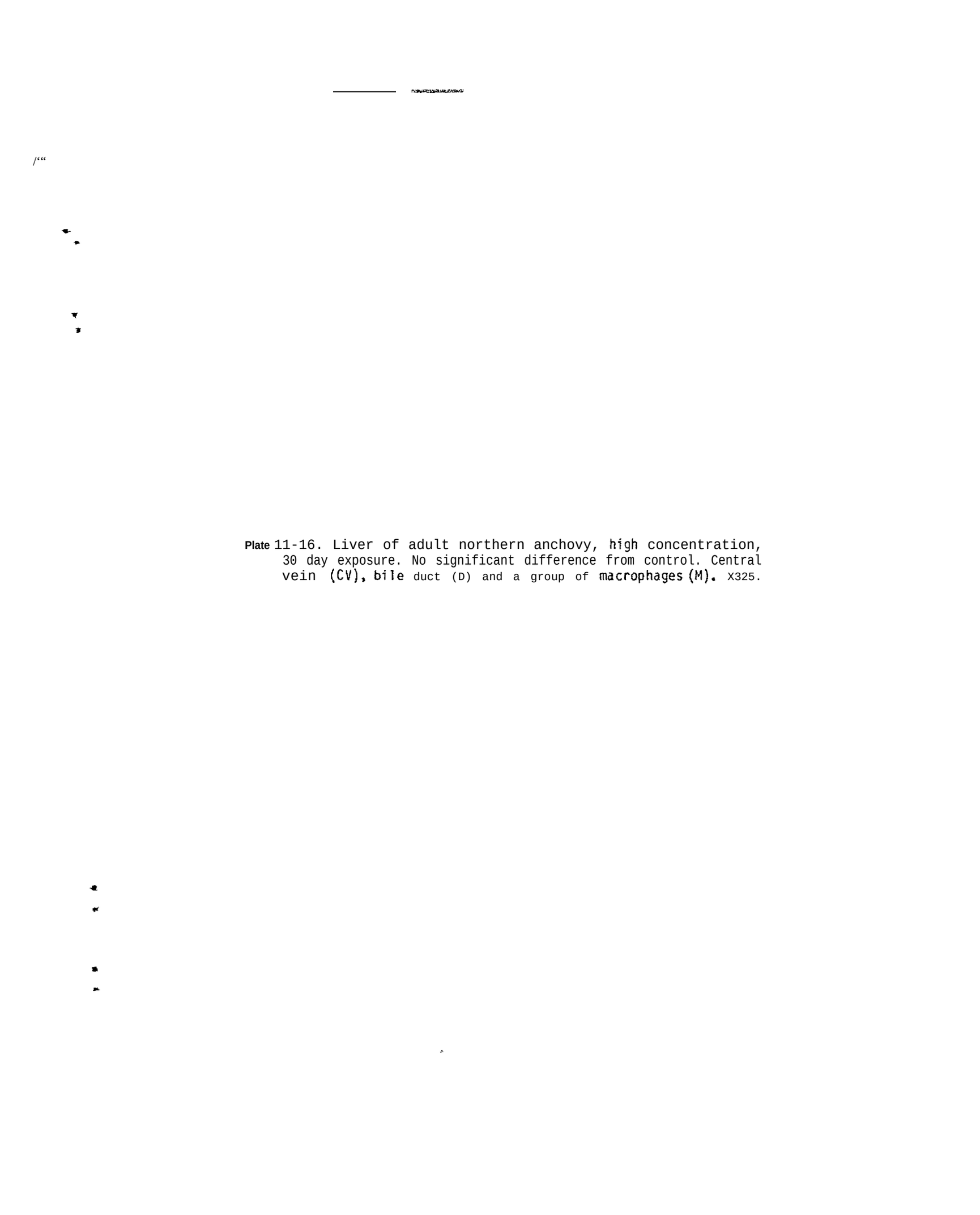


Plate 11-16. Liver of adult northern anchovy, high concentration, 30 day exposure. No significant difference from control. Central vein (CV), bile duct (D) and a group of macrophages (M). X325.

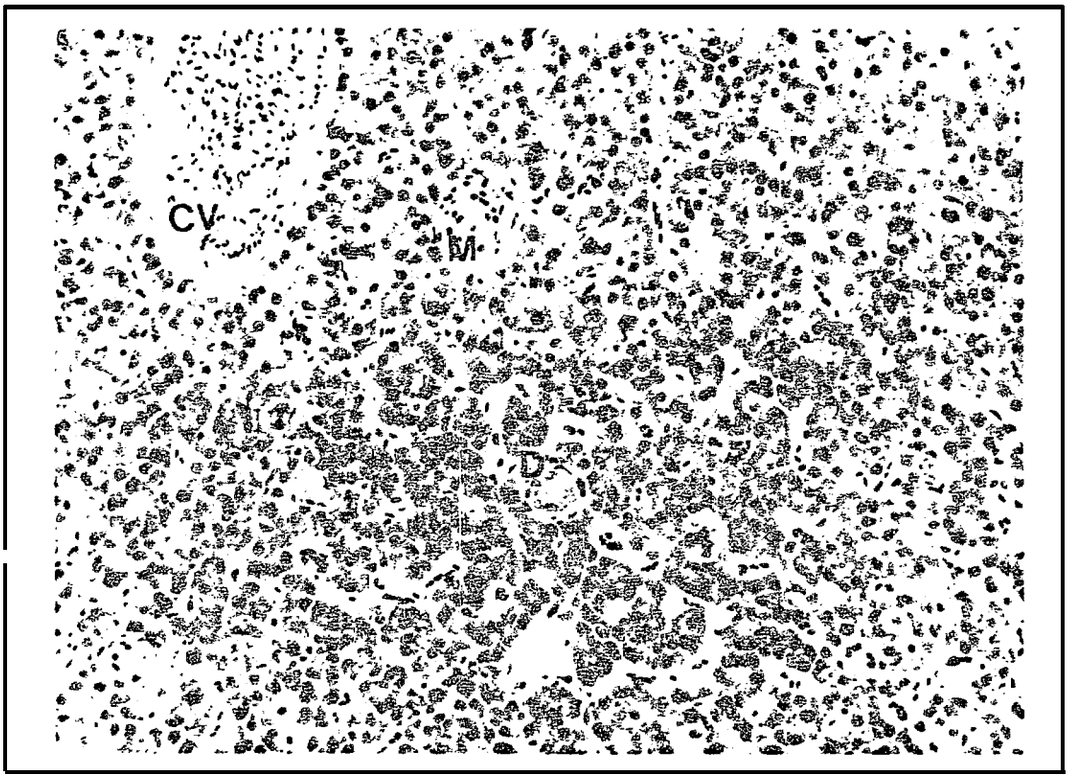

CV
M
D

Plate 11-17 (a-b). Liver of adult northern anchovy from the control group (a) and medium (b) concentration after 60 days of exposure.

a. Liver of adult northern anchovy, control concentration, 60 day exposure. X325.

b. Liver of adult northern anchovy, medium concentration, 60 day exposure. No significant difference from control. X325.

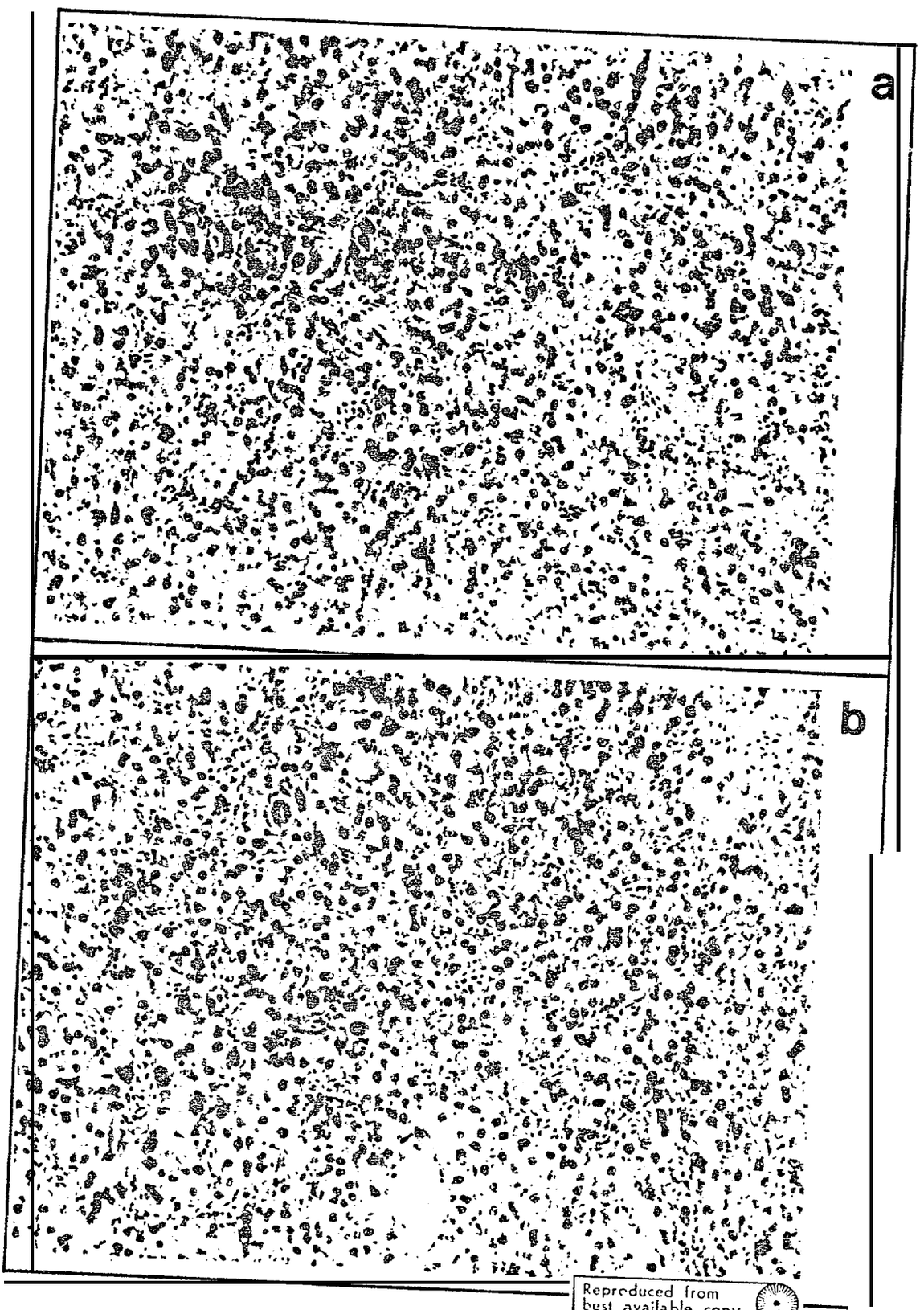


Plate 11-18 (a-b). Liver of adult northern anchovy from the control group (a) and medium (b) concentration after 90 days of exposure.

a. Liver of adult northern anchovy, control concentration, 90 day exposure. X325.

b. Liver of adult northern anchovy, medium concentration, 90 day exposure. No significant difference from control. X325.

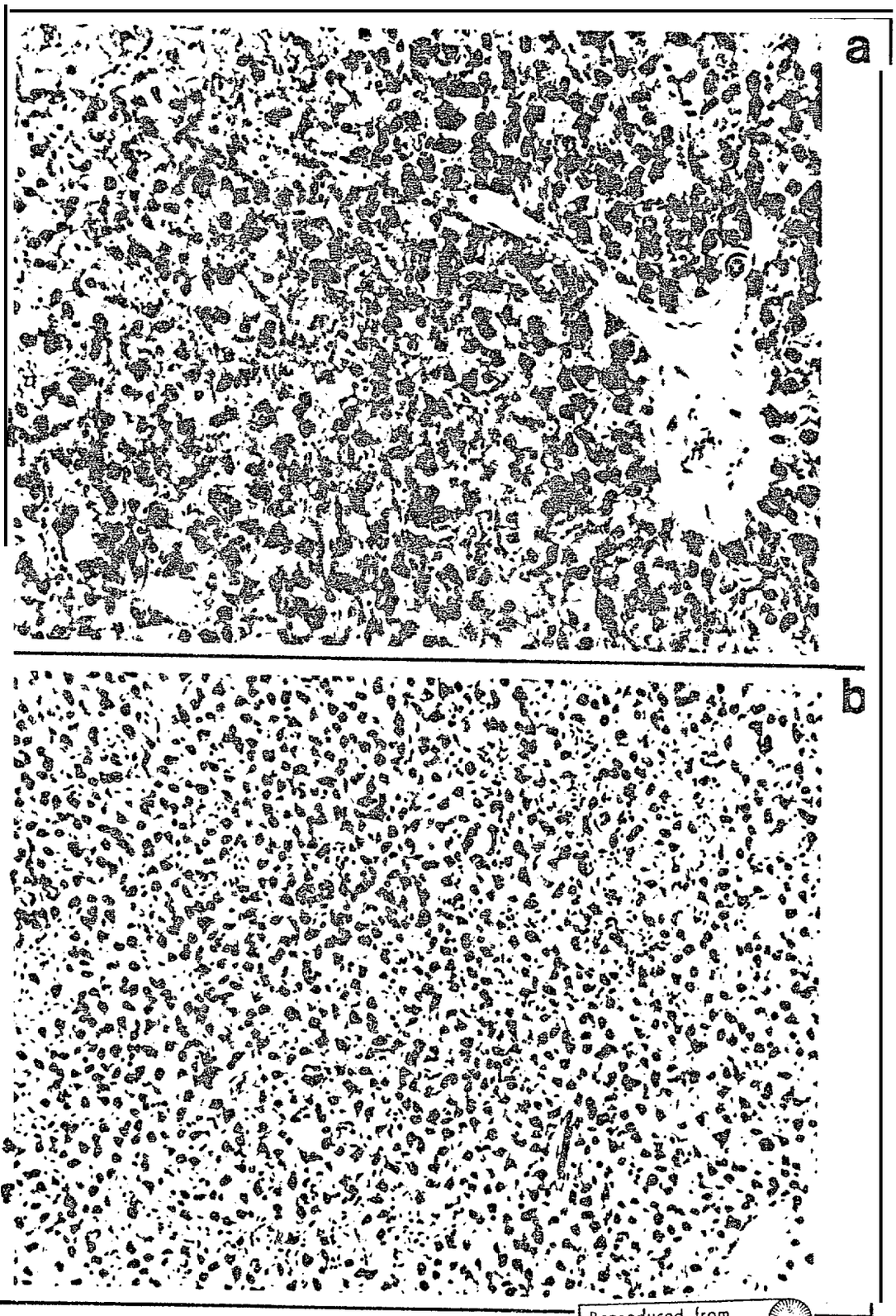


Plate 11-19 (a-b). Liver of adult northern anchovy from the control group (a) and low (b) concentration after 120 days of exposure.

a. Liver of adult northern anchovy, control concentration, 120 day exposure. X325.

b. Liver of adult northern anchovy, low concentration, 120 day exposure. No significant difference from control. X325.

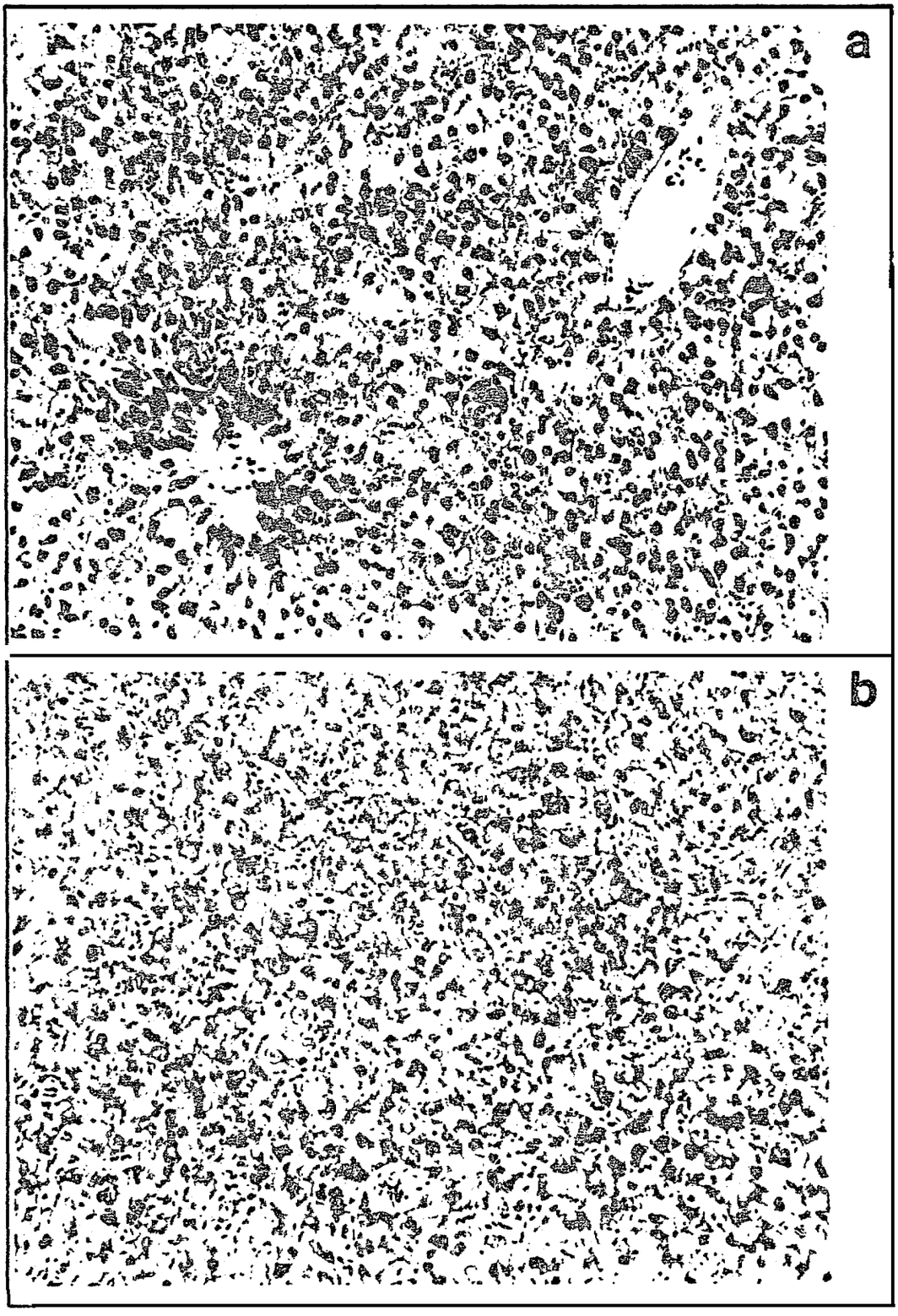

a
b

Plate 11-20. Heart of adult northern anchovy, control concentration, 30 day exposure. Myocardium has outer compact layer (C) and spongy portion (S). Control was not significantly different from experimental. X325.

Plate 11-21. Mucosa of fundus of stomach of adult northern anchovy, high concentration, 30 day exposure. No significant difference from control. X325.

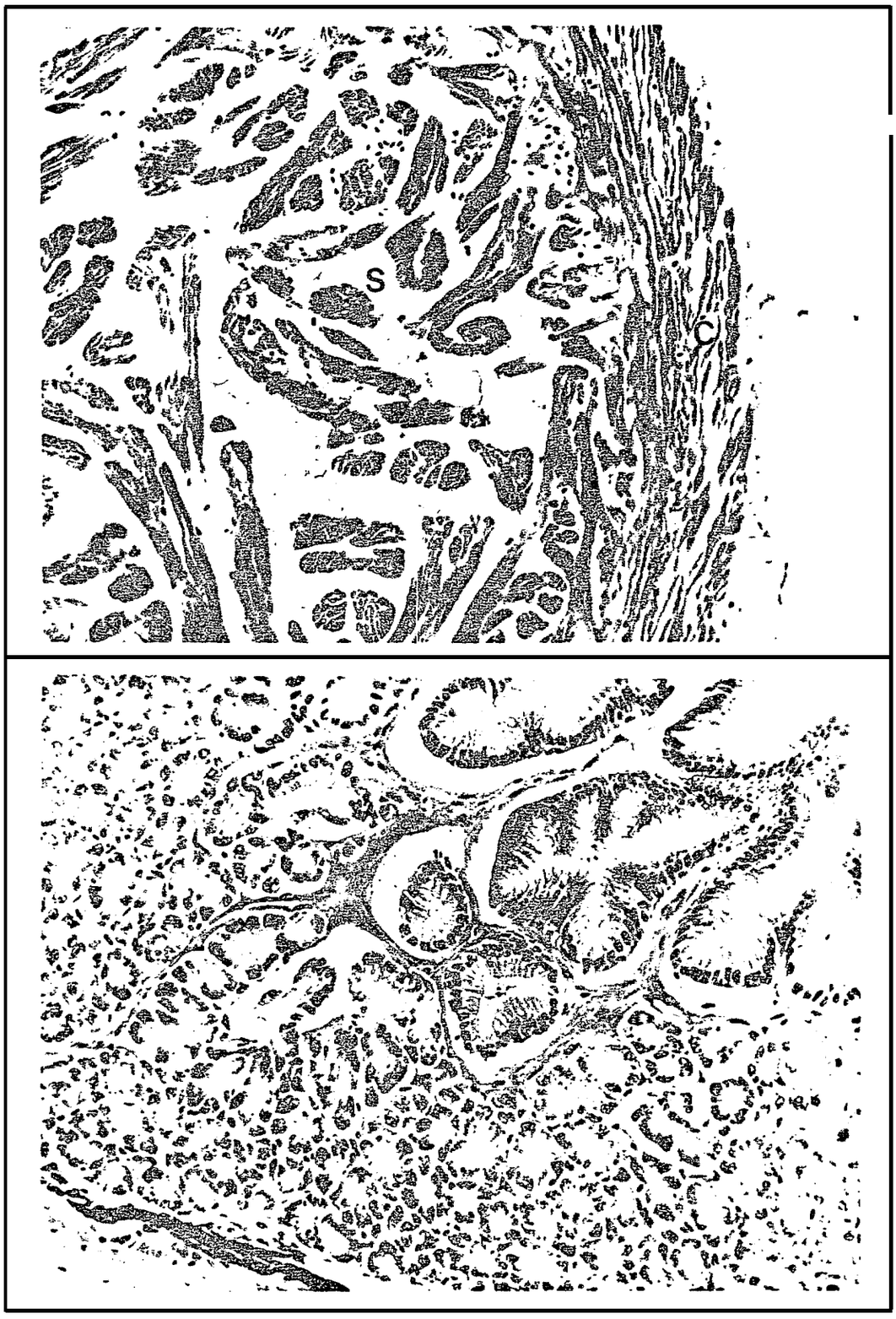

S
C

Plate II-22

a. Pyloric caecum (C), pancreatic acinar tissue (P) of adult northern anchovy, control concentration, 30 day exposure. X325.

b. Pyloric caecum (C), pancreatic duct (D), and pancreatic acinar tissue (P) of adult northern anchovy, high concentration, 30 day exposure. No significant difference from control. X325.

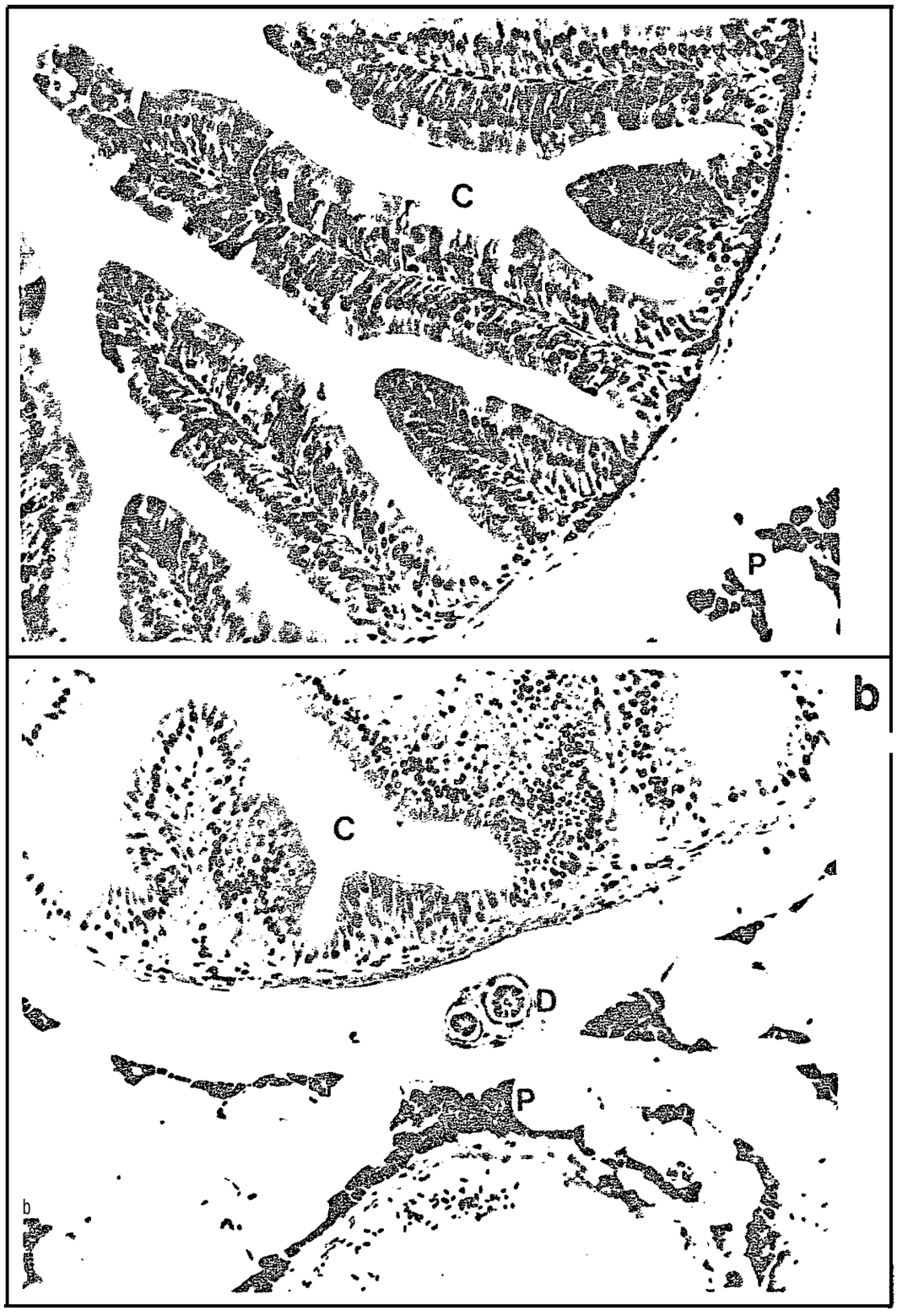
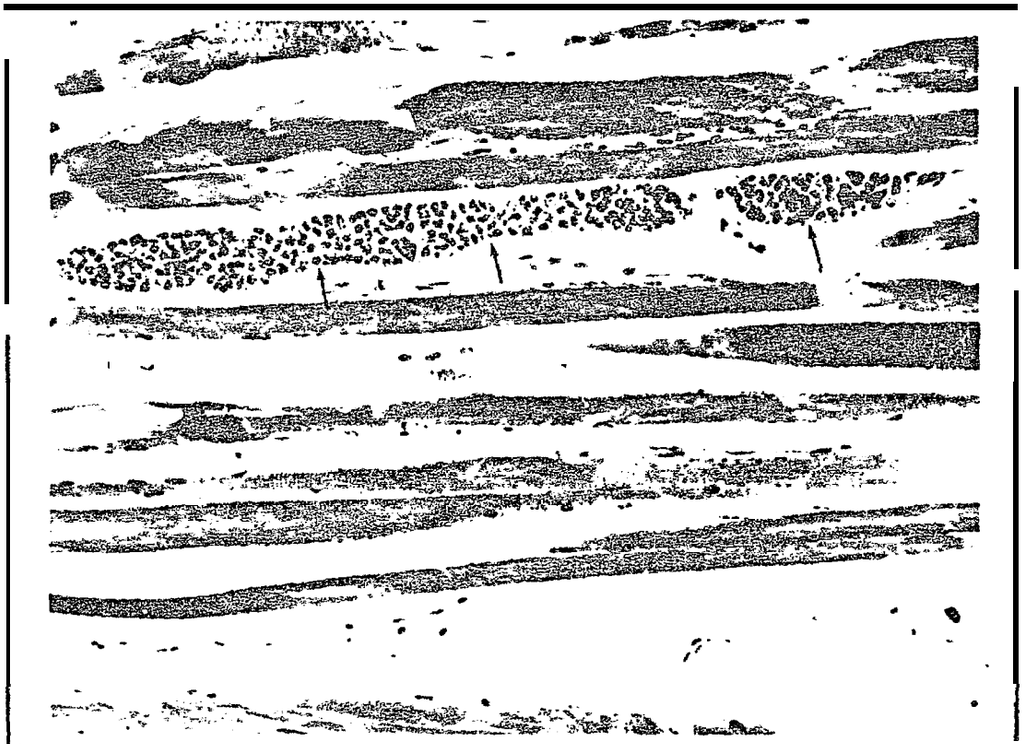

C
P
b
C
D
P
b

Plate 11-23. Skeletal muscle cells of adult northern anchovy, high concentration, 30 day exposure. Note incidental parasitization by Sporozoa (arrows). No significant difference from control. X325.

Plate 11-24. Testis of adult northern anchovy, control concentration, 30 day exposure. No significant difference between controls and experimental. X325.



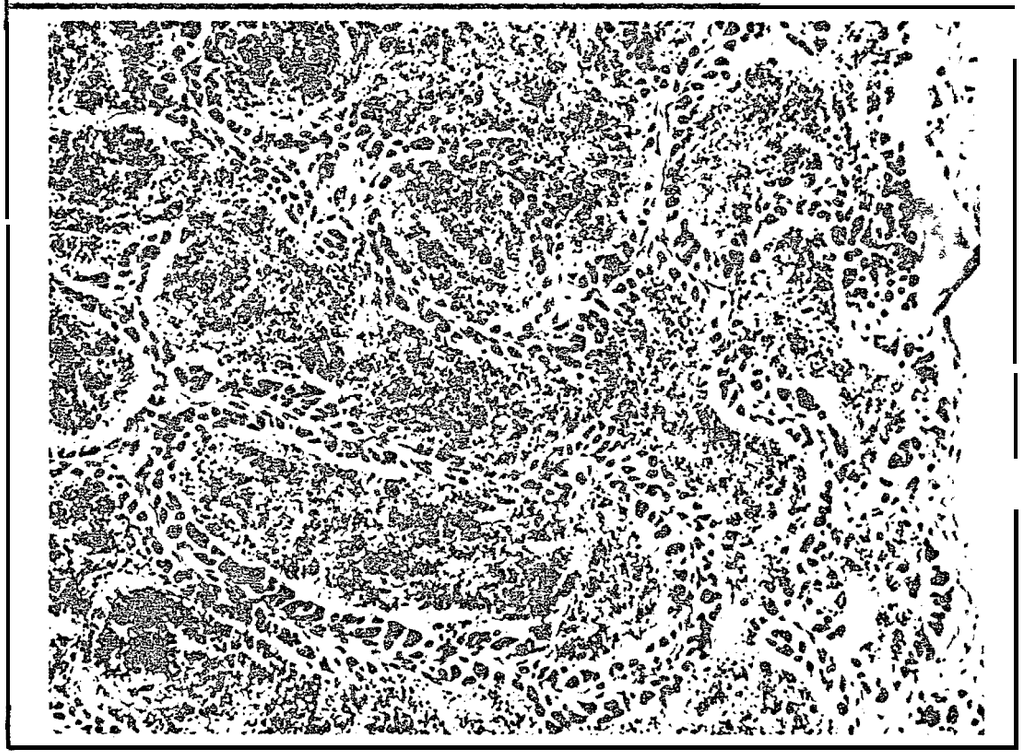


Plate II-25. Ovary of adult northern anchovy, control concentration, 120 day exposure. No significant difference between control and experimental. X325.

Plate 11-26. Brain of adult northern anchovy, control concentration, 120 day exposure. Section through optic tectum or mesencephalon. No significant difference between control and experimental groups. Neurons indicated by arrows. X325.

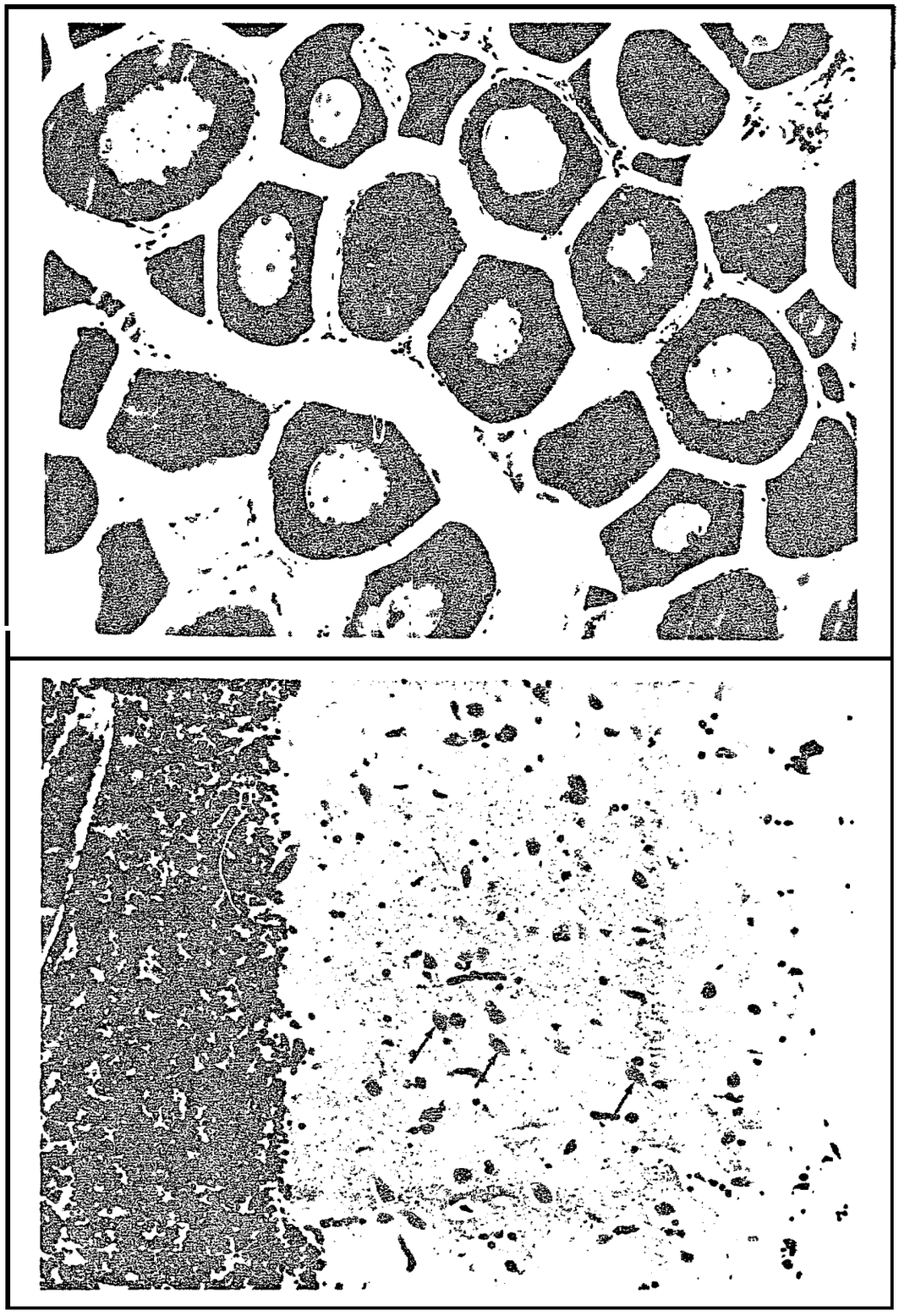


Plate II-27. Retina with optic nerve (O), control concentration, 120 day exposure. No significant difference between control and experimental groups. X325.

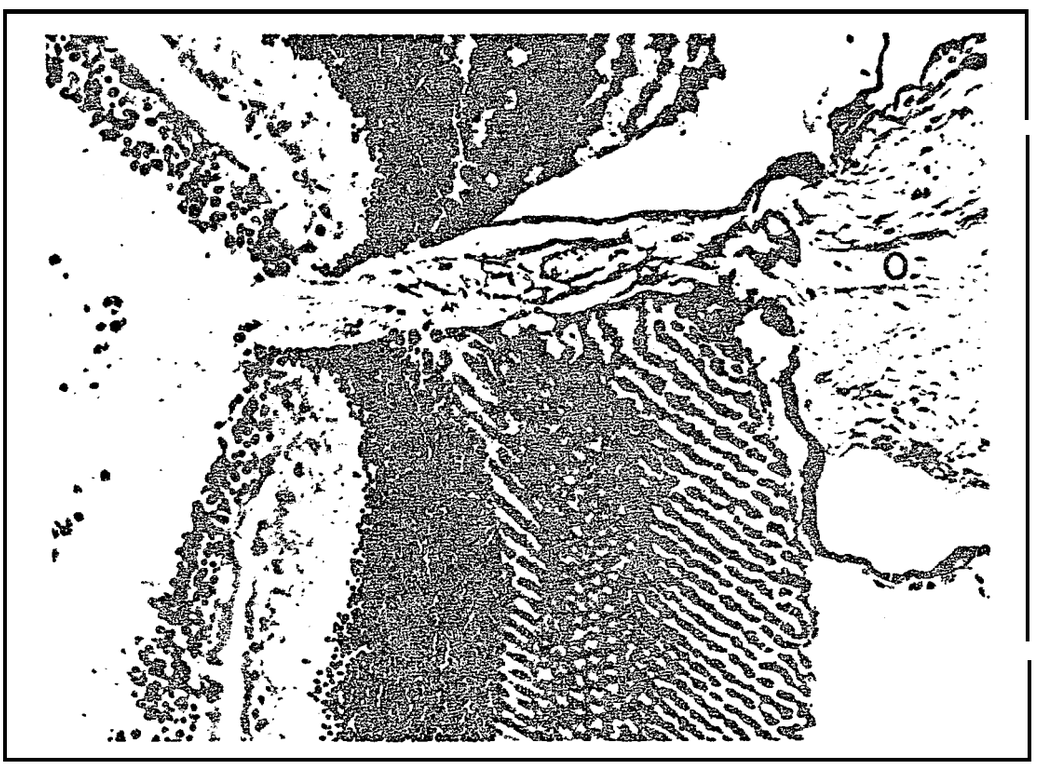


Table II-77. Survivorship of Mytilus californianus embryos exposed to control seawater and test solutions of WSF crude oil for 72 hrs (initial 332±17). Standard errors are shown in parentheses.

Concentration	Mean Number Surviving[a]
Control	296.17 (3.83)[b]
Low	295.00 (4.15)[b]
Medium	287.33 (5.21)[b]
High	246.33 (8.66)[c]

a Survivorship significantly different ($p < 0.05$) among groups; K-W ANOVA.

b,c Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

Table II-78. Mean number of delayed metamorphosis mussel larvae and mean percent deformed veligers in control and experimental exposure regimes developing from embryos exposed for ca. 72 hrs. Standard errors are shown in parentheses.

Concentration	Mean Number Delayed Metamorphosis Larvaea	Mean Percent Deformed Larvae[a]
Control	3.67 (0.92)[b]	1.40 (0.25)[b]
Low	3.50 (1.28)[b]	1.15 (0.40)[b]
Medium	2.33 (0.95)[b]	2.24 (0.365)[b]
High	11.67 (2.4)[c]	9.69 (1.65)[c]

a Metamorphosis significantly different ($p < 0.05$) among groups; K-W ANOVA.

b,c Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

an analog to the SNK multiple comparison test showed that significantly more ($p < 0.05$) embryos exposed to the high concentration test solution exhibited delayed metamorphosis than in the control, low, or medium exposure regimes (Table II-78). Control, low, and medium exposure regimes contained similar numbers of trochophores and were not significantly different.

The percentage of embryos that developed into veligers without shells or with deformed shells was determined for each replicate after the 72 hr experimental period. Mean percentages of abnormal veligers ranged from 1.15 to 9.69 individuals per replicate for low and high concentration exposure regimes, respectively. A K-W ANOVA based on rank transformed data showed there was a significant difference ($p < 0.05$) in the number of deformed larvae found among control and all experimental concentration regimes. Multiple comparison tests by an analog to the SNK multiple comparison test revealed that embryos exposed to the high concentration test solution displayed significantly more ($p < 0.05$) developmental abnormalities than control, low, or medium exposure animals (Table II-78). No significant difference in the number of deformed animals were found among control, low, or medium exposure animals.

Table II-79. Mean shell length and thickness (microns) of veligers which developed from M. californianus embryos exposed to WSF-test solutions for 72 hrs. Standard errors are shown in parentheses.

Concentration	Mean Shell Lengths	Mean Shell Thickness[a]
Control	122.20[b] [0.79]	12.40[b] (0.36)
Low	113.60[c] (1.05)	6.27[c] (0.45)
Medium	103.73[d] (1.15)	5.53[c] (0.24)
High	96.67[e] [2.20]	3.80[d] (0.38)

a Significantly different ($p < 0.05$) among groups; K-U ANOVA.

b,c,d,e Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

Microscopic examination and measurement of metamorphosed larvae (i.e. maximum shell length and thickness measured on 15 veligers per exposure regime) revealed differences in shell length and thickness (Table II-79 and Plate II-28a-d; data appendix). Mean shell length ranged from 97 to 122 microns and shell thickness from 4 to 12 microns for high concentration and control larvae, respectively. A K-W ANOVA performed on shell length and thickness data from control and

exposed animals showed that significant differences ($p < 0.05$) existed in these parameters. An analog to the SNK multiple comparison test was used to determine which groups of test organisms differed significantly in these shell features. Analysis of the shell length data indicated that control veliger shells were significantly larger ($p < 0.05$) than those of veligers developed from embryos exposed to the low, medium or high concentration test solution (Table II-79). Shell lengths of veligers from the low concentration were significantly larger ($p < 0.05$) than those from the medium concentration and both groups were significantly larger ($p < 0.05$) than animals exposed to the high concentration exposure regime.

Microscopic examination of whole-mounted larvae revealed that the shell-thickness of veliger larvae exposed to test concentrations was thinner and less consistent in thickness than in control animals (Plate Ii-29a-d). Multiple comparison test results for shell thickness data are shown in Table II-79. The tests demonstrated that shell thickness in control veligers was significantly greater ($p < 0.05$) than that of veligers developing from embryos exposed to any of the three test solution concentrations. Shell thickness of veligers from low and medium concentrations was not significantly different; however, both groups of veligers possessed shells that were significantly thicker than those of larvae from the high concentration.

5) Histopathology. Histological examination of selected organs in embryos was not feasible during the program, since an extensive research and development effort was required. However, whole organism mounts were prepared and subjected to microscopic examination. These results were presented in Section 4.a.4), Development.

b. Larval Experiments

1) Hydrocarbon Exposure. Hydrocarbon exposure data for the California mussel larval experimental studies are presented in Table II-80. Background hydrocarbon concentration in the control water for larval Mytilus californianus experiments was approximately 4.4 µg/l. Over the course of the larval M. californianus study, the mean hydrocarbon content in the low exposure level experimental waters was 14.2 µg/l. This value was found to be significantly different than that of the control water at the 95% significance level (Student's t-test). The corrected mean hydrocarbon exposure value for the medium level test solution was 80.5 µg/l, and the high test solution averaged 559 µg/l. In all cases, hydrocarbon concentrations corrected for contaminant levels approached the target values, and mean values between low, medium and high exposure levels were shown to be statistically different at the 95% significance level.

Table II-80. Hydrocarbon exposure concentrations for California mussel larval experiment.

Exposure Level	Hydrocarbon Concentration (µg/l)[a] Volatile Fraction	Extractable Fraction[b]	Total[c]
Control	0.9+ 0.7	3.5+3.7 [8.73.31	4.4+ 3.7 [9.6+ 5.3]
Low	3.6: 1.6	10.6+6.1 [18.4+3.3]	14.2+ 6.8 [22.0+ 4.1]
Medium	33.7+22.2	46.8+43.4 [54.2+43.9]	80.5+50.0 [87.8149.5]
High	227+147	332+163 [336+165]	559+89 [563+91]

a Where appropriate, data are reported as the mean +1 standard deviation.

b Extractable hydrocarbon val . are reported corrected for the presence of diethyl tet, an, rofuran and uncorrected values are also presented in brackets.

c Total hydrocarbon values represent the sum of the average daily volatile fraction plus the weekly extractable fraction. Values are al so reported both corrected and uncorrected for diethyltetrahyd rofuran.

2) Hydrocarbon Tissue Burden. Radioactive tracer uptake was used as an indirect measure of hydrocarbon bioaccumulation. Upon termination of replicates at days 7, 11, 15, and 24,

Plate II-28(a-d). Representative photographs of whole-mounted recently metamorphosed veligers from control and test solution exposure regimes. Developing embryos exposed for approximately 72 hrs under static conditions. The treated larvae are relatively small and have an irregular outline due to abnormal shell formation. Shells have irregular thick and thin zones.

a. Veliger from control exposure regime shows shell is thicker than any shells of exposed specimens. Note also that the shell is more uniform in thickness. The thinnest portion of the perimeter is indicated by a single arrow, the thickest by a double arrow **(X500).**

b. Irregular shell from low concentration. Note thin portion of shell perimeter indicated by single arrow, thick segment by double arrow (X500).

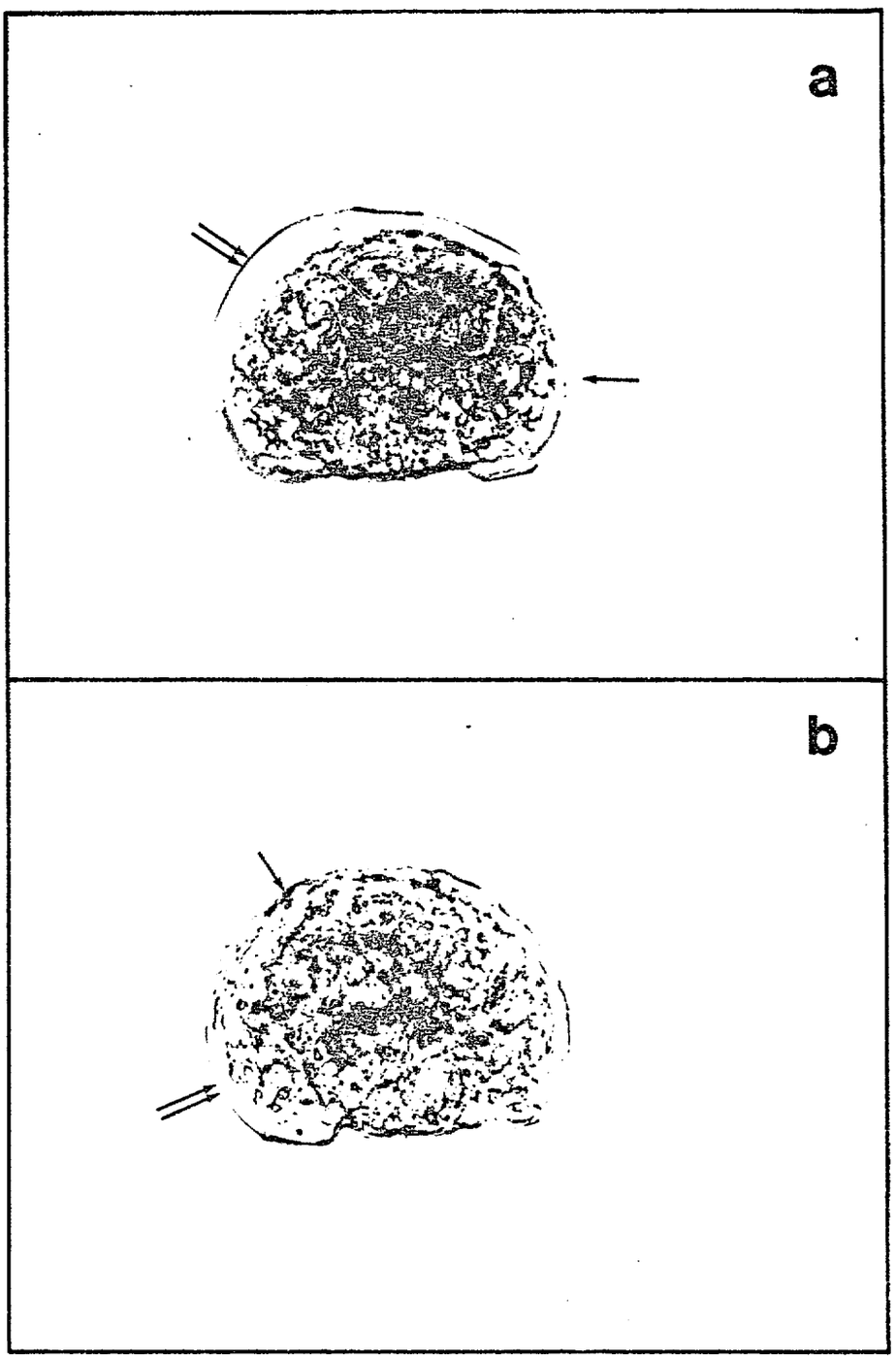

a
b

Plate II-28(a-d). (Cent)

c. Note uneven shell thickness in medium concentration. Thin portions of shell perimeter indicated by single arrow, thick segment by double arrow (X500).

d. Uneven shell thickness is observed in high concentration in both larvae pictured. Note thin portions of shell perimeter indicated by single arrows, and relatively thick zones by double arrows (X500].

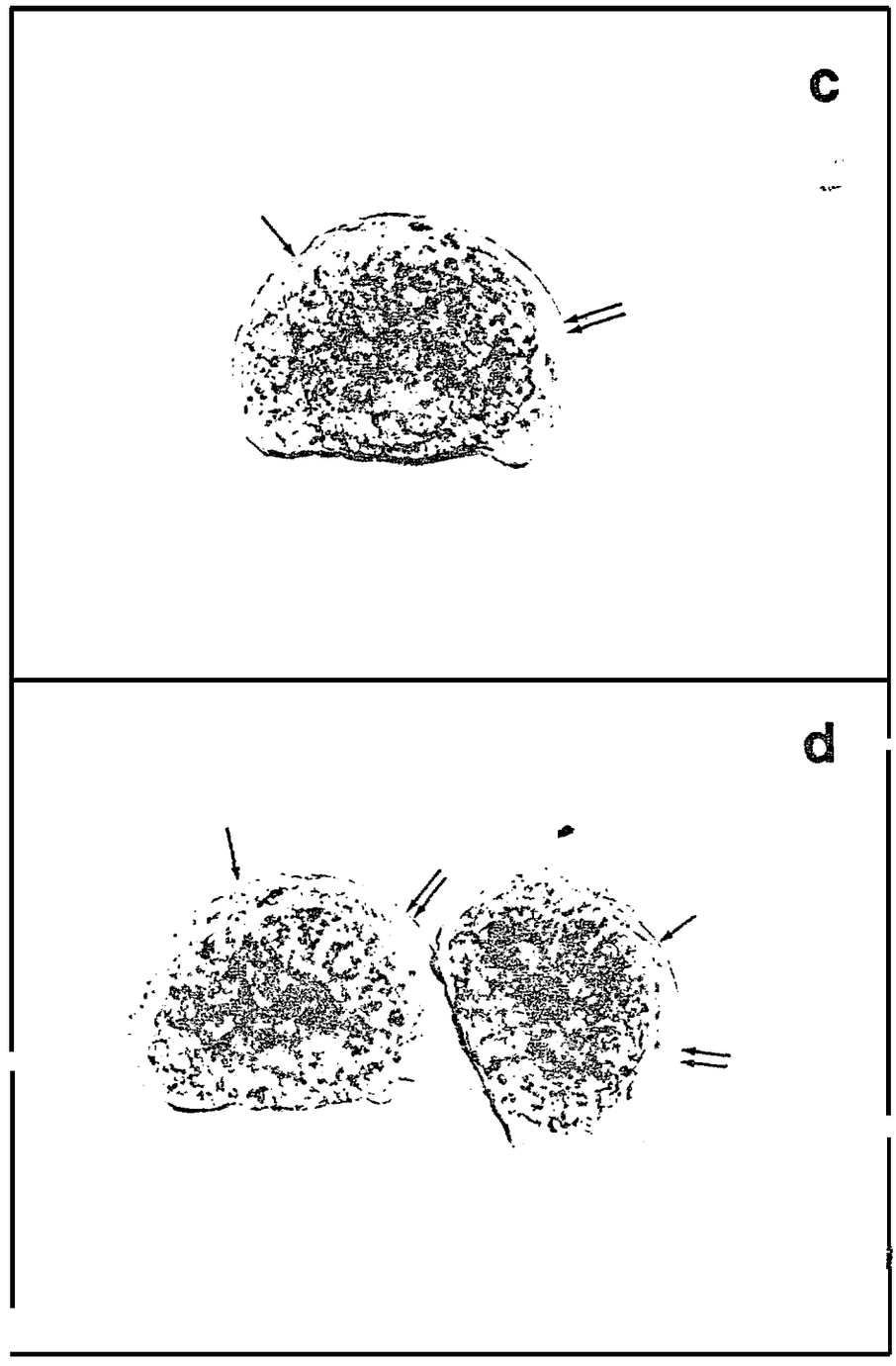

c
d

Plate II-29(a-d). Representative photograph of whole-mounted veligers from the control (a), low (b), medium (c), and high (d) concentrations after 7 days. The focal plane in each picture demonstrates relative equality and uniformity of shell thickness in all four specimens. In (c) the optical section is not perfectly aligned, giving the false impression of a defective shell. The arrow indicates inner border of shell in its ventral region. All shells and larvae appear normal (x500).

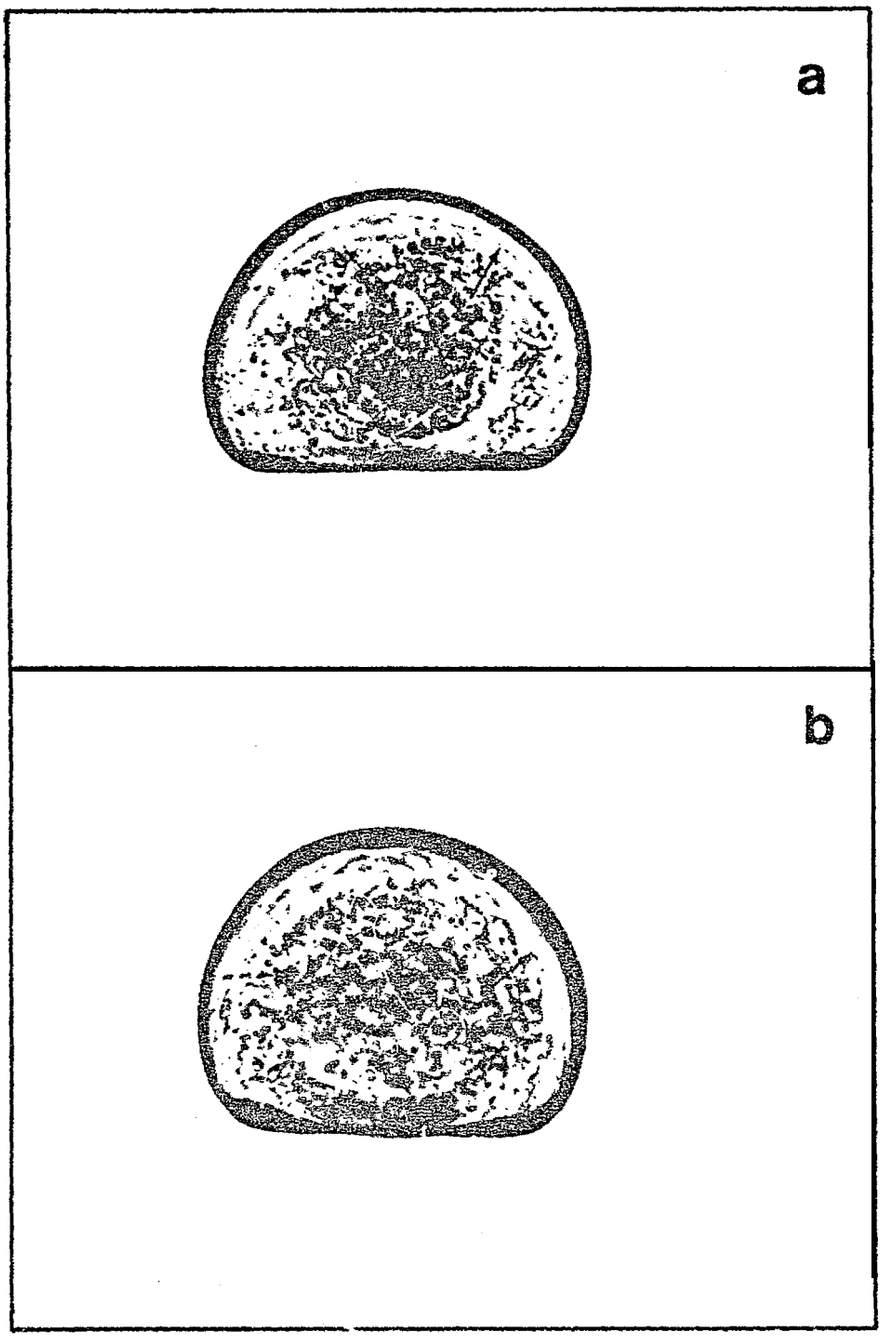

a
b

Plate II-29(a-d). (Cent)

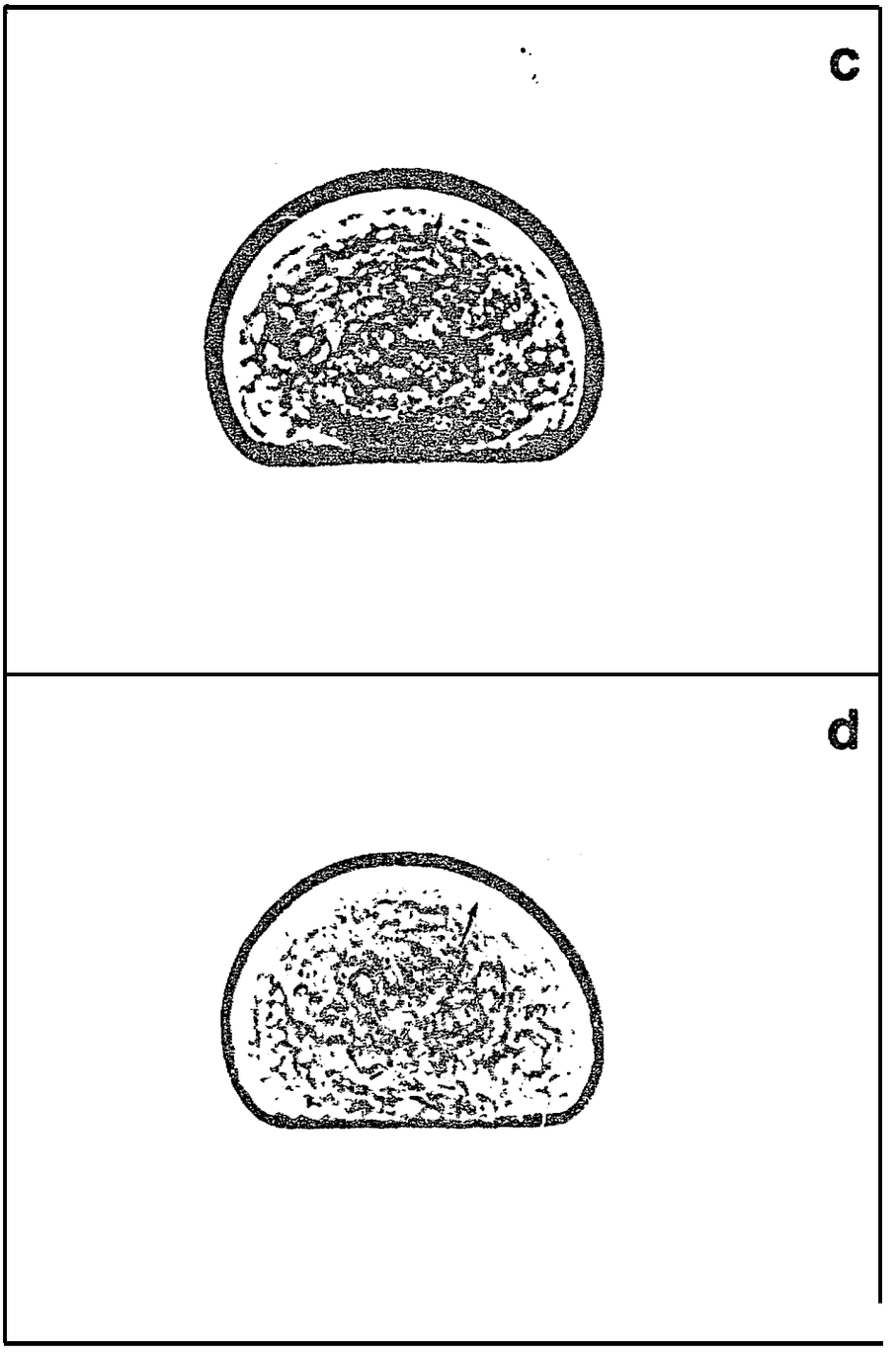

c
d

Table 11-81. Mean radioactive tracer uptake (counts/minute/veliger) in M. californianus veligers at 7, 11, 15, and 24 days. Standard errors are shown in parentheses.

Concentration	Exposure Period (Days) 7[a]	11[a]	15[a]	24.3
Control	1.97 (0.21)[b]	4.29 (0.34)	6.10 (0.60)[b]	13.37 (0.49)[b]
Lou	1.88 (0.09)[b]	- -	14.87 (2.73)[c]	31.13 (3.06)[c]
Medium	2.79 (0.27)[c]	- -	25. 17 (2.03)[d]	54.7 (e)[c]
High	6.15 (1.69)[c]	21.83 (1.53)	- -	

a Tracer uptake significantly different ($p < 0.05$) among groups; K-U ANOVA.

b,c,d Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

e Two replicates combined because of low number of surviving larvae.

radioactive tracer counts were made on subsamples of 50 veligers from each replicate (Table 11-81). Counts per larva were calculated from total counts per minute and larval numbers data. Tracer uptake for larvae exposed to the high concentration exposure regime was only monitored at days 7 and 11, since high mortality necessitated termination by day 11. No evaluation of tracer uptake was made at day 11 in low or medium concentrations.

Radioactive tracer uptake on day 7 was determined for veligers from three replicates in each test solution exposure regime and the control. Mean tracer uptake ranged from 1.87 to 6.15 cpm per larva in control and high concentrations, respectively. A K-W ANOVA based on ranked uptake data showed that tracer uptake by M. californianus veligers was significantly different ($p < 0.05$) among the control and all test solution concentrations (Table 11-81). A non-parametric analog of the SNK multiple comparison test indicated that no significant difference ($p > 0.05$) in radioactive tracer uptake existed between control and low, or between medium and high concentration experimental animals. However, there was significantly greater ($p < 0.05$) bioaccumulation of radioactive tracer in medium and high concentration larvae compared to low and control animals (Table 11-81).

On day 11, elevated mortality in the high concentration exposure larvae necessitated termination of this experimental regime. Subsamples of larvae were obtained from control aquaria to provide data for comparison with high concentration animals. Mean tracer uptake for larvae in the high concentration exposure regime was 21.83 cpm, while uptake for control animals was 4.29 cpm. A K-W ANOVA showed that veligers exposed to the high concentration regime contained significantly ($p < 0.05$) higher levels of radioactive tracer.

Veliger bioaccumulation of radioactive tracer was measured on day 15 by making counts on subsamples of larvae from three replicates in each test concentration and control exposure regime. Mean counts ranged from 6.10 to 25.17 cpm for control and medium concentration larvae, respectively. A K-W ANOVA indicated that larval tracer uptake levels were significantly different among the control, low, and medium concentration exposure regimes. A multiple comparison test with the SNK analog revealed significantly higher ($p < 0.05$) levels of radioactive tracer had been incorporated into veligers exposed to the medium concentration compared with control and low concentration larvae. In addition a significant difference ($p < 0.05$) in tracer uptake was found between larvae exposed to control seawater and the low test solution concentration.

Radioactive tracer uptake was measured on veligers subsampled from ail remaining replicates upon termination of the experiment on day 24. Larvae

(e.g. 50) were obtained from each of three replicates in the control and low concentration exposure regimes. High mortality in the medium concentration necessitated a combination of two replicates to obtain adequate numbers of veligers for uptake determination. Tracer levels ranged from 13.37 to 54.7 cpm/larva for control and medium exposure animals, respectively. A K-W ANOVA on ranked data indicated that significant differences ($p < 0.05$) existed for larval tracer uptake between the control, low, and medium exposure regimes. Multiple comparison tests with the SNK analog showed that tracer uptake by veligers in low and medium concentration exposure regimes was significantly ($p < 0.05$) higher than in control animals. No significant difference in tracer levels of larvae exposed to low and medium test solution concentrations was detectable.

3) Survivorship. Larval survivorship was monitored through counts of aliquots subsampled from replicate aquaria. Veliger mortality was high in the control and all WSF test solution concentrations. These results, in part, reflect the high initial mortality which occurs in nature. In spite of the natural mortality, a clear inverse relationship between veliger survival and test solution concentration existed. Veligers exposed to the high concentration test solution survived for 11 days. Control, low, and medium concentration veligers survived for 24 days, but with significant reduction in numbers.

On day 7 of the larval experiment, three replicates each from the control, low, medium, and high concentration test solutions were randomly terminated to evaluate larval mortality. Estimates of survivorship for each replicate were based on three aliquots (1 ml each) subsampled from each aquarium, The mean density of surviving larvae per replicate aquarium was used as a measure of survivorship, since all aquaria started with the same density (21.7 veligers/ml) at the beginning of the experiment. The mean number of surviving larvae ranged from 1.08/ml in the high concentration to 14.73/ml in the control (Table 11-82). A K-W ANOVA based on ranked survival data indicated that veliger mortality on day 7 was significantly different ($p < 0.05$) in the control and three test. solution concentrations. A SNK multiple comparison test showed that larval mortality in both the medium and high concentrations was significantly higher than that in the low concentration and control seawater ($p < 0.05$). Larval survivorship was not significantly different in the control and low concentration test regimes, nor was there a detectable difference in survivorship of animals exposed to medium and high concentration test solutions.

The density of veligers in the high concentration exposure regime had decreased sharply by day 11, therefore, all high concentration replicates were terminated at this time. To provide a basis for statistical comparison, three aliquots

Table II-82. Mean number per milliliter of surviving M. californianus larvae (veligers) at 7, 11, 15, and 24 days. Standard errors are shown in parentheses.

Concentration	Exposure Period (Days) 0[a]	7[b]	11[b]	15[b]	24[b]
Control	21.7	14.7 (0.70)[c]	6.5 (1.00)	6.9 (1.20)[c]	0.21 (0.03)[c]
Low	21.7	14.6 (0.58)[c]	- -	5.7 (0.72)[c]	0.18 (0.03)[c]
Medium	21.7	11.87 (0.60)[d]	- -	1.30 (0.34)[d]	0.004 (0.002)[d]
High	21.7	1.08 (0.12)[d]	0.04 (0.02)	- -	

a Initial density (number/ml) of larvae/replicate.

b Tracer uptake significantly different ($p < 0.05$) among groups; K-W ANOVA.

c,d Groups designated by different letters significantly different ($p < 0.05$) from all other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

(1 ml each) were drawn from each control aquarium (total of six aquaria). A K-W ANOVA showed that there was significantly greater mortality ($p < 0.05$) in the high concentration aquaria than in the control.

Three replicates from each test concentration and the control were terminated on day 15. Veliger densities were determined from three aliquots (i ml each) drawn from each aquarium. Larval densities ranged from 1.30/ml to 6.9/ml in the medium concentration and control, respectively. A K-W ANOVA based on ranked survival data indicated that mortality by day 15 was significantly different ($p < 0.05$; Table II-82) among test specimens. A SNK multiple comparison test showed that larval mortality in the medium concentration was significantly higher ($p < 0.05$) than in the control or low concentrations. Further, no significant difference in larval survivorship was found between the control and low concentration exposure regime.

Veliger densities in all aquaria had declined substantially by day 24. At this point, all remaining replicates in the control, low, and medium concentration regimes were terminated. Larval densities for the control and low concentrations were determined from three 500 ml aliquots. Extremely low densities of veligers in the medium concentration necessitated examination of the entire volume (approximately 3 liters) in each replicate aquarium (i.e. three replicates). Larval densities ranged from 0.004/1111 to 0.21/ml in the medium and control aquaria, respectively. A K-W ANOVA on the ranked data revealed a significant difference ($p < 0.05$) in survivorship between exposure regimes. A SNK multiple comparison analog test showed that significantly higher mortality ($p < 0.05$) occurred in the medium concentration exposure regime than in control or low concentrations. No detectable difference in survivorship could be found for larval densities in the control and low concentration regimes.

4) Development. Veliger growth in the control ant! all three test solution exposure regimes was monitored by measuring length and height at days 7, 11, 15, a . 24. Measurements were made on approximately 20 larvae from each replicate terminated at a particular time interval (e.g. 7, 11, 15, 24 days). Except for day 11, three replicates from each exposure regime were terminated. Elevated mortality in the high concentration exposure regime necessitated termination of all six replicates on day 11. To provide a basis for evaluating larval growth data from the high exposure regime on day 11, 20 animals were subsampled and measured from six control replicates.

Length and height measurements obtained on larvae were treated in the identical statistical manner. In all cases, statistical test results for length comparisons were identical to those for height. For example, when a statistically significant length difference was found to exist between larvae from control and high exposure regimes, the same was true for height differences. Therefore, the statistical results presented below describe size (i.e. general term applicable to length or height) comparisons. However, Table II-83 and Figures 11-23 and II-24 present both length and height data.

Veliger shell length and height were measured on day ?' (Table II-83). Mean lengths ranged from 108.63 to 118.53μ for larvae from the high and low exposure concentration regimes, respectively (Table II-83). Mean heights ranged from 78.66 to 90.68μ, again for the high and low exposure regimes, respectively (Table 11-83). A K-W ANOVA based on size data indicated that significant differences ($p < 0.05$) existed among control veligers and those

Table II-83. Mean shell measurements (length [L] and [H] in microns) for M. californianus veligers made on days 7, 11, 15, and 24 of the larval experiment. Standard errors are shown in parentheses.

	Exposure Period (Days)							
	7a		11[a]		15[a]		24a	
Concentrate on	L	H	L	H	L	H	L	H
control	115.56 (1.70)[b]	88.20 (0.87)[b]	122.03 (0.17)	83.43 (0.42)	119.37 (0.33)[b]	89.98 (0.58)[b]	129.80 (0.33)[b]	101.05 (0.58)5
Low	118.53 (0.66)[c]	90.68 (0.88)[c]			125.62 (0.33)[c]	96.08 (0.58)[c]	126.68 (0.33)[c]	97.23 (0.88)[c]
Medium	117.10 (1.67)[c]	90.12 (0.58)[c]			115.02 (0 00)[d]	81.25 (0.33)[d]	115.54 (0.50)[d]	89.23 (0. 00)[d]
High	108.63 (0.67)[d]	78.66 (0.88)[d]	110.66 (0.24)	79.95 (0.20)				

[a] Length (or height) of control veligers and those exposed to test concentrations significantly different ($p < 0.05$) among groups; K-id ANOVA.

[b,c,d] Groups designated by different letters significantly different ($p < 0.05$) from al 1 other groups; non-parametric analog to SNK multiple comparison test. Groups with same letter not significantly different.

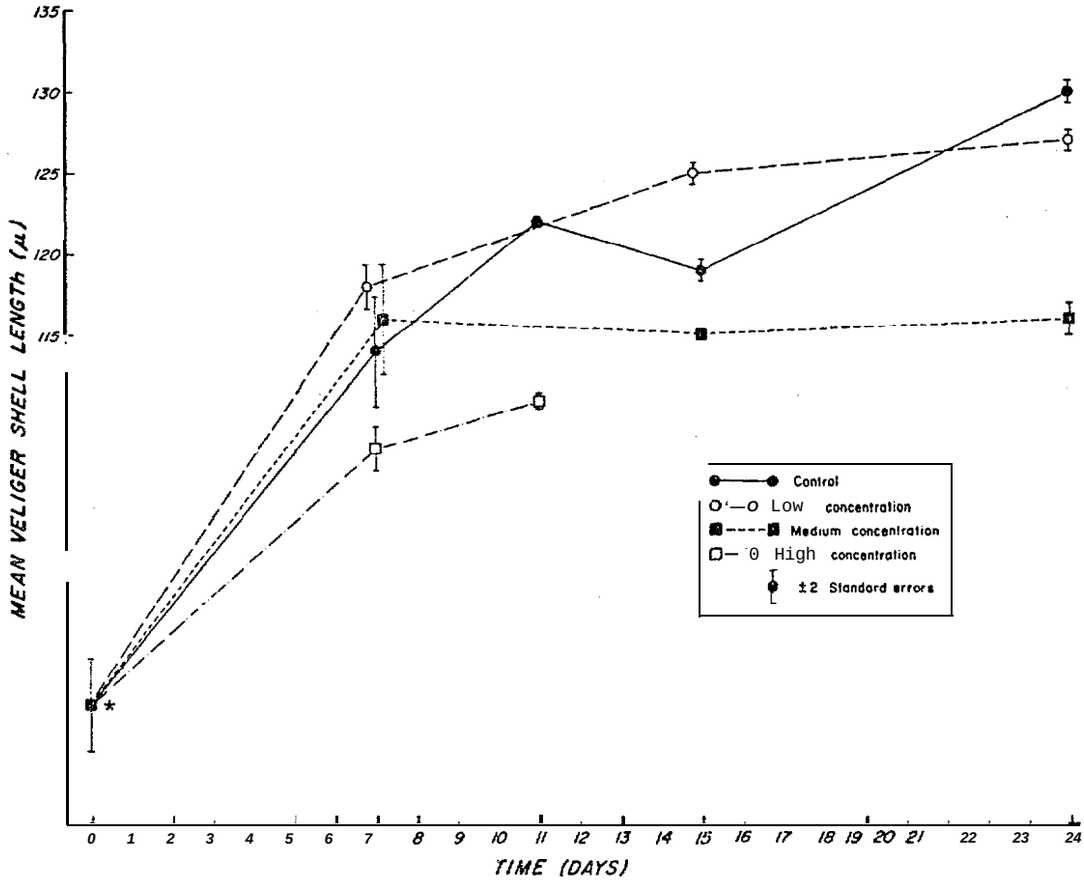


Figure II-23. Mean shell length (microns) of California mussel veligers following 7, 11, 15, and 24 days of test solution exposure. * = Initial shell length 92.3±5.5 microns.

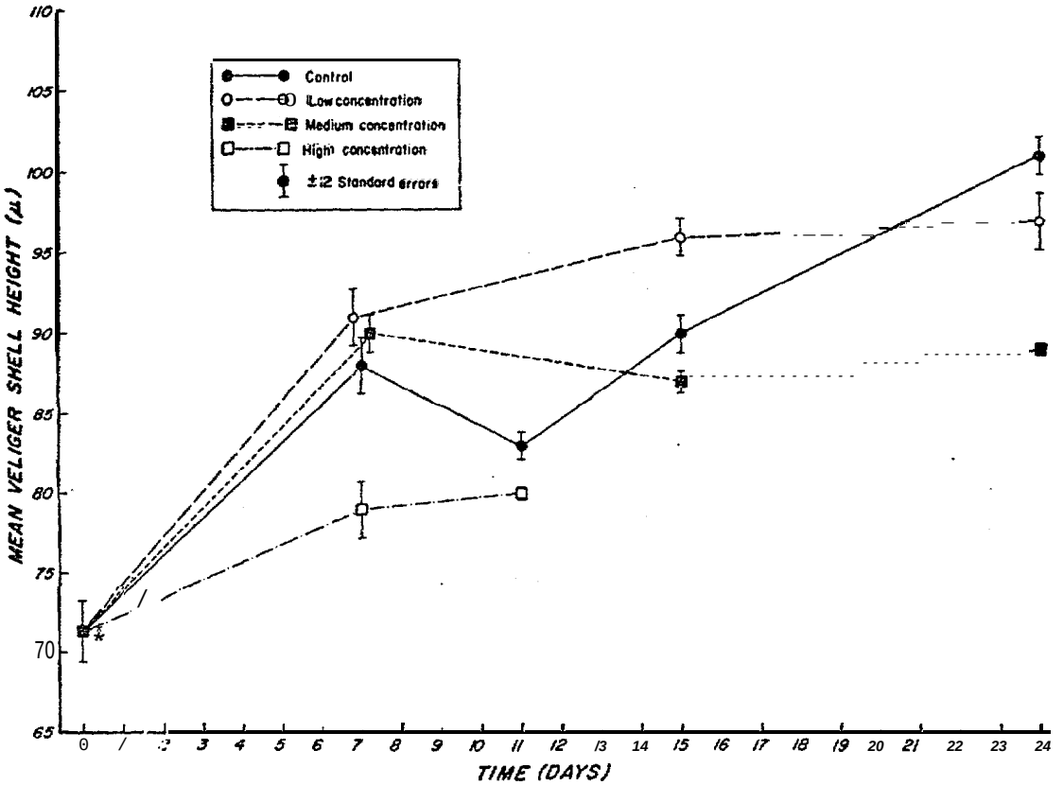


Figure II-24. Mean shell height (microns) of California mussel veligers following 7, 11,15, and 24 days of test solution exposure. *=Initial shell height 71.3±37 microns.

exposed to all test solution concentrations. . Multiple comparisons of ranked size data revealed that veligers from the low and medium concentration test regimes were significantly ($p < 0.05$) larger than those from the control or high concentration regimes. No significant difference in size was detectable between low and medium exposure larvae. In addition, control larvae were significantly ($p < 0.05$) larger than veligers exposed to the high concentration.

Elevated veliger mortality in the high concentration test exposure necessitated termination of that exposure regime on day 11. Mean shell length measurements for control and high concentration larvae were 122.03 and 110.66E, respectively. Mean shell heights for control and high exposure larvae were 83.43 and 79.95μ, respectively. A K-W ANOVA of the size data indicated that control animals were significantly larger ($p < 0.05$).

Veliger size measurements on days 15 and 24 showed similar relationships between exposure regimes. Mean length measurements ranged from 115.02 to 125.62μ on day 15, and from 115.54 to 129.80μ cn day 24 for larvae exposed to medium and control test solution concentrations, respectively. Mean shell heights on day 15 ranged from 87.25 to 96.08μ for medium and low exposure larvae, respectively, and on day 24 from 89.23 to 101.05μ for medium exposure and control larvae, respectively. K-W ANOVAS performed on data from both time periods revealed significant size differences existed between control larvae

and those exposed to the low and medium exposure regimes. Multiple comparison testing using the SNK analog on ranked data showed that on day 15 veligers exposed to low concentrations of test solution were significantly larger ($p < 0.05$) than control and medium concentration veligers. Control larvae were also significantly larger ($p < 0.05$) than those exposed to the medium concentration test solution. Multiple-comparisons on day 24 veliger size data produce" slightly different results. Control and low exposure larvae were significantly larger ($p < 0.05$) than veligers exposed to the medium concentration of test solution. However, control larvae were significantly ($p < 0.05$) larger than low exposure animals.

Whole-mounted M. californianus veligers were examined for microscopic evidence of delayed or abnormal development. Shell thickness of 15 randomly selected larvae was measured from each concentration during each time interval (i.e. day 7, 15, and 24). Plates II-29 through II-32 illustrate larvae from the various groups. Measurements of shell thickness (Table II-84) for veligers developing in WSF test solutions were not significantly different from controls for any time interval.

5) Histopathology. Histological examination of organs in veligers was not feasible during this program, since an extensive research and development effort was required. However, whole organism mounts were prepared and subjected to microscopic examination. These results were presented in Section 4.a.4), Development.

c. Adult Experiments

1) Hydrocarbon Exposure. Hydrocarbon exposure data over the course of the 18 week adult California mussel chronic toxicity study are summarized in Table 11-85). Corrected hydrocarbon levels in the control waters averaged 5.0 µg/l and those from the low level test solution averaged 12.7 µg/l. Statistical analysis of these data indicates that the mean hydrocarbon levels are different at the 95% significance level (Student's t-test). Mean hydrocarbon levels of 48.1 and 413 µg/l were observed over the course of this study for the medium and high level dosing solutions. As evidenced by Table 11-85, all exposure levels were relatively consistent and close to target values.

Table 11-84, Veliger shell thickness of larvae exposed **to control seawater** and three test **solution concentrations. Standard** errors **are shown in parentheses. No significant differences between shell characteristics** for any time **period (i.e. 7, 15, or 24 days).**

	Shell Thickness		
	Exposure Period (Days)		
Concentration	7	15	24
Control	11.73 (0.51)	11.73 (0.45)	12.07 (0.25)
Low	11.73 (0.64)	12.20 (0.45)	11.47 [0.36]
Medium	11.60 (0.47)	11.87 (0.43)	*
High	11.80 (0.46)	*	*

• Insufficient numbers of survivors for evaluation.

2) Hydrocarbon Tissue Burden. Selected tissues from sacrificed adult mussels were analyzed for petrogenic hydrocarbon content upon termination of the adult experiment. Tissues selected for analysis included muscle (retractor and adductor), gill, testis, ovary and digestive gland. Each tissue was examined for parent petrogenic hydrocarbon content and replicate samples of each tissue were analyzed. All samples represented composites of tissue from a number of individual organisms. Tissue burden characterizations were performed on samples from background (i.e. random subsample of organisms collected for the experiments), control and dosed (low, medium and high level) organisms.

Plate II-30. Representative photograph of whole-mounted veliger from the high concentration exposure regime after 11 days. Shell appears normal (X500).

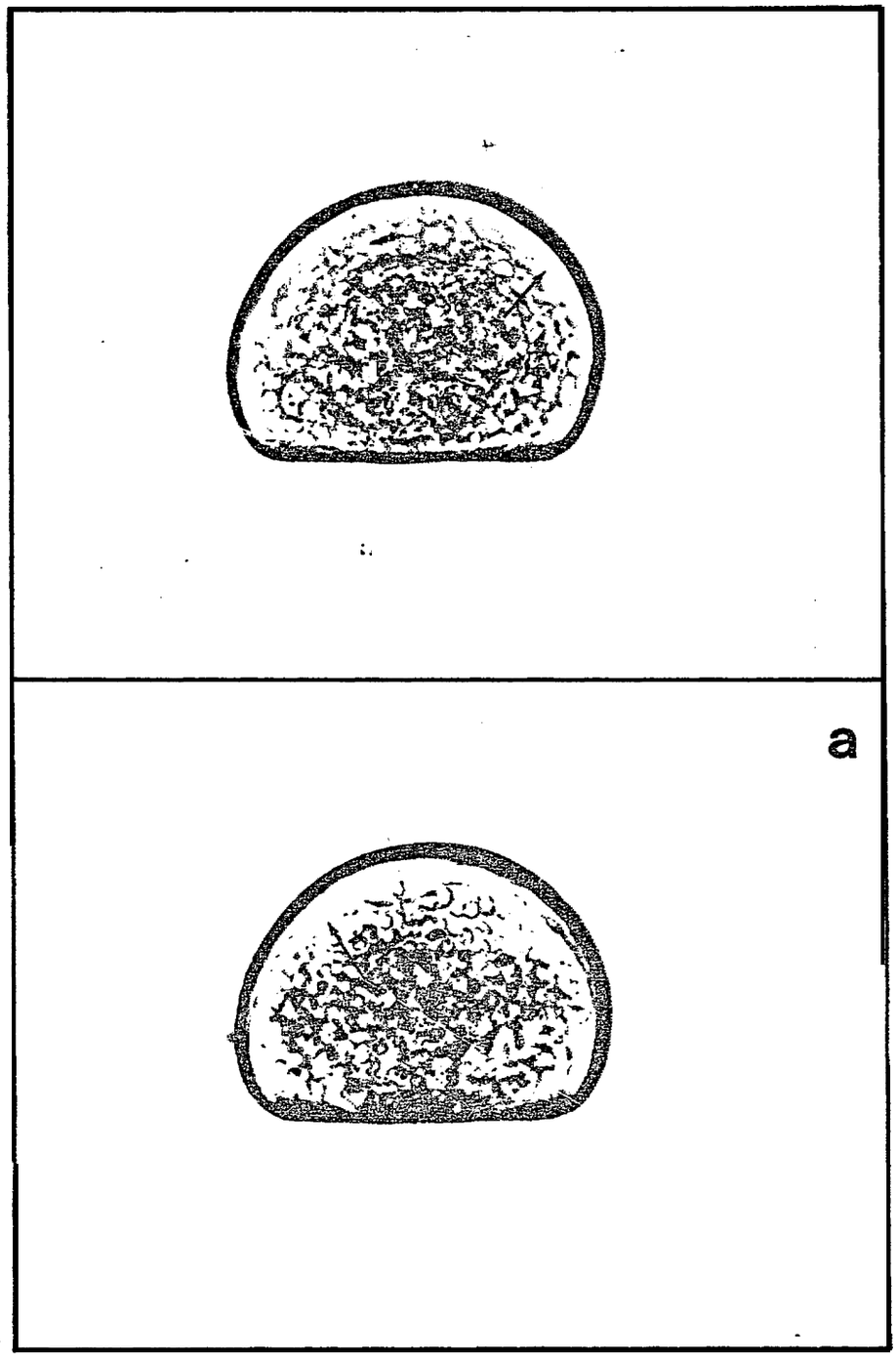

a

Plate 11-31 (a-c). (Cent)

b. Low concentration with two arrows indicating thickness of shell in vicinity of dorsal ligament.

c. Medium concentration showing inner limit of ventral aspect of shell by arrow.

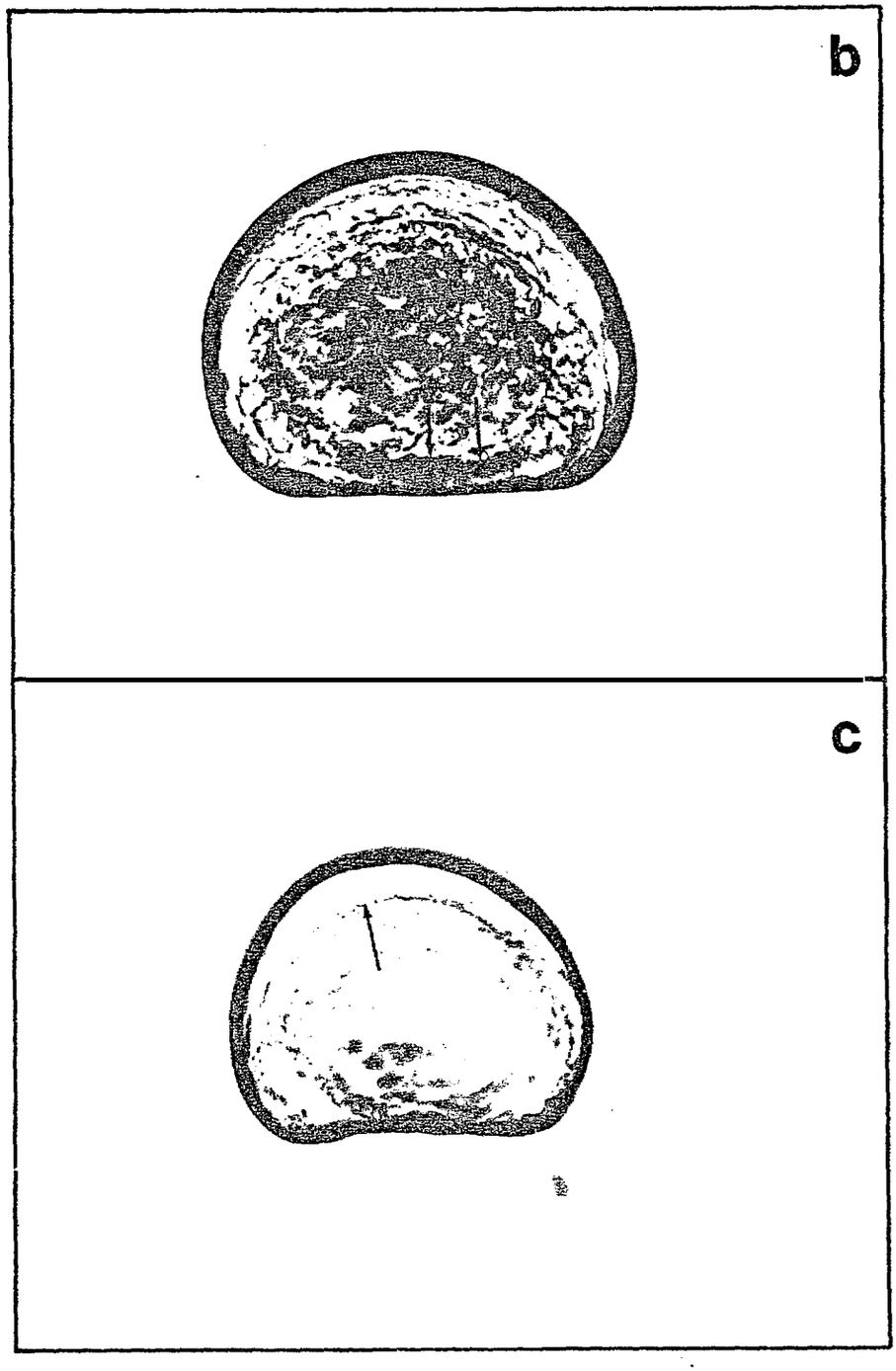

b
c

Plate II-32(a-b). Representative photographs of whole-mounted veliger from the control (a) and low (b) concentration exposure regimes after 23 days. Illustrates two unusual larvae not conforming to general pattern of their groups.

a. Control larva has abnormally irregular shell in comparison to cohorts. X500.

b. Veliger larva from group treated at low concentration has relatively thick shell in comparison to cohorts. X500.

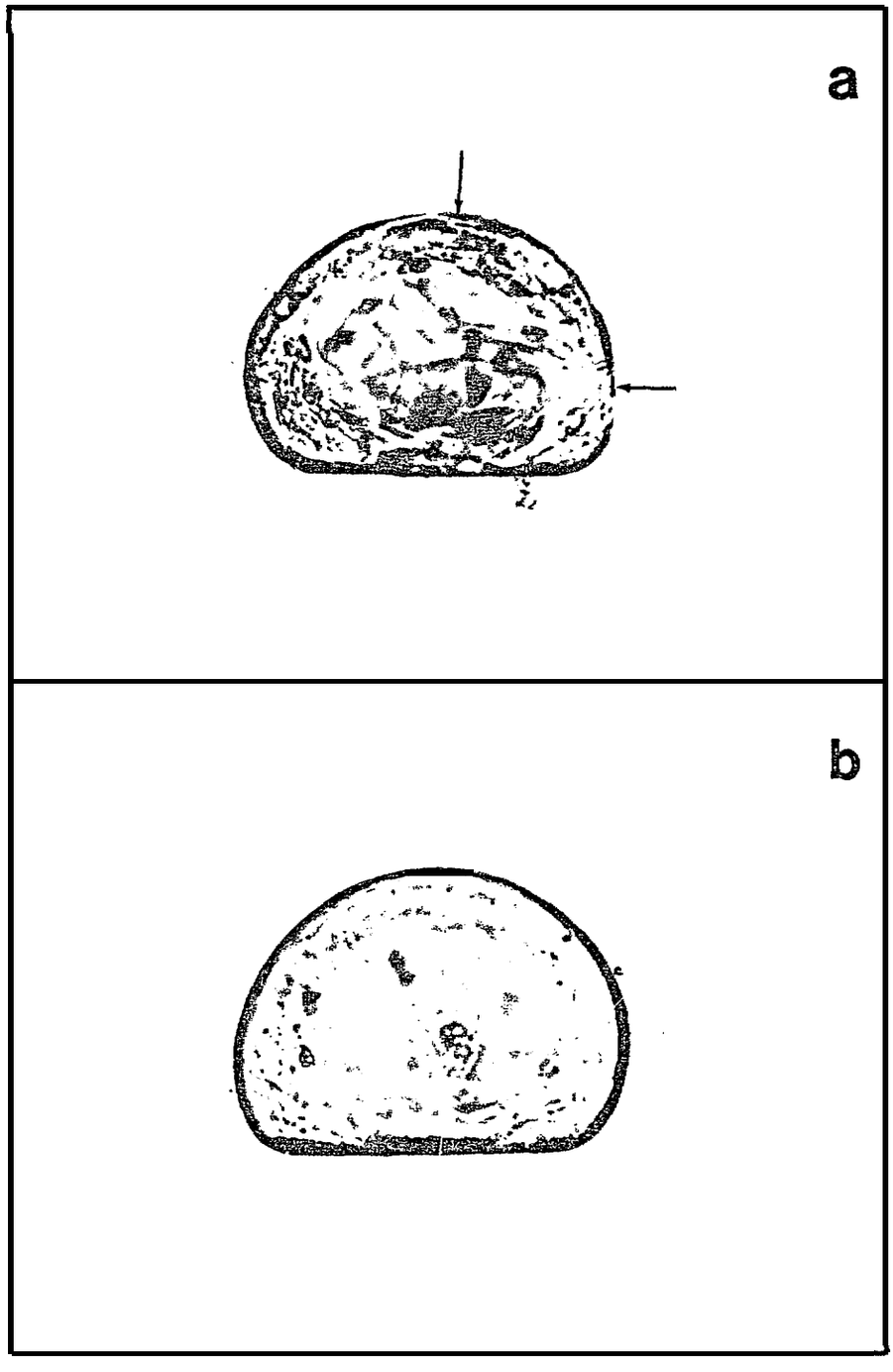

a
b

Table II-85. Hydrocarbon exposure concentrations for adult California mussel experiment.

Exposure Level	Hydrocarbon Concentration (ug/l)[a] Volatile Fraction	Extractable Fraction[b]	Total[c]
Cent rol	0.5±0.3	4.5±2.6 [12.2±4.5]	5.0± 2.6 [12.7± 4.5]
Low	8.0±13.6	4.7±2.5 [12.5±4.4]	12.7± 9.2 [20.5± 9.81
Medium	38.5±19.7	9.5±5.7 [17.93.31	48.1±21.5 [56.4±21.5]
High	253±90	161±102 [170±99]	413±137 [423±136]

[a] Where appropriate, data are reported as the mean ±1 standard deviation.

[b] Extractable hydrocarbon values are reported corrected for the presence of diethyl tetrahydrofuran and uncorrected values are also presented in brackets.

[c] Total hydrocarbon values represent the sum of the average daily volatile fraction plus the weekly extractable fraction. Values are also reported both corrected and uncorrected for diethyl tetrahydrofuran.

Petrogenic hydrocarbon tissue burden data are presented in tabular summary format in this section, and detailed characterization data are presented in the data appendix. Hydrocarbon data are grouped into four categories, including benzenes, naphthalenes, thiophenes and furans, and phenanthrenes. Each of these categories is composed of the parent hydrocarbon and its alkyl-substituted homologs. The total tissue burden is also given in each table and represents the summary of the four categories plus any additional petroleum derived hydrocarbons which were identified.

Results of tissue burden characterizations for muscle (retractor and adductor) and gill tissues are presented in Tables II-86 and II-87, respectively. As Table II-86 indicates, no hydrocarbons of petroleum origin were detected in muscle tissue extracts from any of the experimental organisms, including background and control animals. Similarly, petroleum hydrocarbons were not detected in background, control and low exposure level extracts from M. californianus gill tissues. However, extracts from gill tissue of medium and high exposure level organisms were found to contain 65 and 77 rig/g of petrogenic hydrocarbons, respectively. In both cases, the tissue burden was composed solely of two isomers of dimethylphenanthrene and mean concentration values showed small standard deviations.

Table II-88 summarizes bioaccumulation data for the digestive gland. The bicaccumulation pattern is similar to that observed for the M. californianus gill tissue, although tissue concentrations were considerably greater. No detectable levels of petrogenic hydrocarbons were found in tissues from background, control and low exposure level organisms. Significant levels of petroleum-derived aromatic hydrocarbons were identified in extracts

Table II-86. Petrogenic hydrocarbon burden in adult California mussel retractor **adductor muscle tissue.**

Toxicant Category[b]	Exposure Level[d] Bkg[c]	Cent rol	Low	Medium	High
Benzenes	ND	MD	ND	NO	ND
Naphthalenes	ND	ND	ND	ND	Nb
Thiophenes and Furans	ND	MD	ND	ND	ND
Phenanthrenes	ND	ND	ND	ND	ND
Total Petrogenic Hydrocarbons Identified	NO	ND	NO	ND	ND

ND = None detected.

a Concentration data reported as mean ±1 standard deviation in ng/g dry weight,

b Each category is composed of the parent compound and alkyl-substituted homologs.

c Bkg = Background tissue sample (tissue sample from organisms collected at the same time as those used for exposure studies)

Table II-87. Petrogenic hydrocarbon burden in adult California mussel gill tissue.

Toxicant Category[b]	Exposure Level[1] Bkg[c]	Control	Low	Medium	High
Benzenes	ND	ND	ND	ND	ND
Naphthalenes	ND	ND	ND	ND	ND
Thiophenes and Furans	ND	ND	ND	ND	ND
Phenanthrenes	ND	ND	ND	65±8	77±8
Total Petrogenic Hydrocarbons Identified	ND	ND	ND	65±8	77±8

NO = None detected.

1 Concentration data reported as mean ±1 standard deviation in ng/g dry weight.

2 Each category is composed of the parent compound and alkyl-substituted homologs.

3 Bkg . Background tissue sample (tissue sample from organisms CO I lected at the same tine as those used for exposure studies) .

from organisms exposed to medium and high level test solutions. In both cases, a variety of naphthalene, thiophene and phenanthrene derivatives were identified. Medium exposure level samples had an average petrogenic hydrocarbon content of 890 rig/g, which was roughly evenly divided among naphthalene, thiophene and phenanthrene derivatives.

Bioaccumulation data for the ovary tissues are presented in Table II-89. The bioaccumulation pattern observed in the California mussel ovary was similar to that seen for the digestive gland. Tissue extracts from background and control organisms showed no detectable levels of petrogenic hydrocarbons. Organisms exposed to the low level test solution contained trace levels of benzene derivatives, while significant tissue burdens were detected at the medium and high exposure levels. Mean petrogenic hydrocarbon tissue burdens of 1059 and 815 rig/g were detected in extracts from organisms exposed to the medium and high level test solutions, respectively. Comparable concentrations of alkyl-substituted benzenes were identified at both exposure levels, but substantial variation existed among other hydrocarbon categories. Thiophene derivatives were the predominant species identified in medium exposure level extracts, while high exposure level organisms showed the greatest accumulation of alkyl-substituted naphthalenes.

Hydrocarbon tissue burden data for male gonad tissue from M. californianus are summarized in Table 11-90. Trace levels of petrogenic hydrocarbons were detected in extracts of testes from organisms exposed to the low level test solution, while no petrogenic hydrocarbons were identified in extracts from background and control animals. At the medium exposure level high concentrations of alkyl-substituted benzenes and naphthalenes were identified, as well as relatively low levels of substituted thiophenes. The total tissue burden at the medium exposure level averaged 675 rig/g.

Testicular extracts from organisms exposed to the high level test solution contained high levels of a full spectrum of petroleum-derived aromatic hydrocarbons. A total of 44 individual petrogenic hydrocarbons were identified, including 17 alkyl benzenes, 12 alkyl naphthalenes, seven derivatives of thiophene and furan, five alkyl phenanthrenes, and three indenes. Mean concentrations of individual hydrocarbons within these groups ranged from 38 rig/g to as high as 1100 rig/g. Alkyl-substituted naphthalenes and thiophenes/furans showed the greatest bioaccumulation, both with average

Table H-88. Petrogenic hydrocarbon burden in adult California mussel digestive tract tissue.

	Exposure Level[1]				
Toxic ant Ca tegory[b]	Bkg[c]	Contr( )	Low	Medi um	Hi gh
Benzenes	MD	ND	NO	ND	ND
Naphtha lenes	ND	ND	ND	94±51	373+86
Thiophenes and Furans	ND	ND	ND	751±59	303±53
Phenanthrenes	ND	ND	ND	45±10	409+59
Total Petrogenic Hydrocarbons Identified	ND	ND	ND	890:100	1084+92

ND = None detected.

1 Concentration data reported as mean +1 standard deviation in ng/g dry weight.

2 Eachcategory is composed of the parent compound and alkyl -substituted homologs.

3 Bkg . Background tissue sample (tissue sample from organisms collected at the same time as those used for exposure studies).

Table II-89. Petrogenic hydrocarbon burden in adult California mussel ovary tissue.

	Exposure Levels				
Toxicant Category[b]	Bkg[c]	Control	Low	Medium	High
Benzenes	ND	ND	*	300+434	240±62
Naphtha lenes	ND	ND	ND	19±33	492±224
Thiophenes and Furans	ND	ND	ND	671±410	53±92
Phenanthrenes	NO	ND	ND	ND	30+53
Total Petrogenic Hydrocarbons identified	ND	ND	*	1059±369	815±245

ND = None detected. * = Trace amount detected.

[a] Concentration data reported as mean +1 standard deviation in ng/g dry weight.

b Each category is composed of the parent compound and alkyl -substituted homologs.

c Bkg = Background tissue sample (tissue sample from organisms collected at the same time as those used for exposure studies).

Table 11-90. Petrogenic hydrocarbon burden in adult California mussel testes tissue.

Toxicant Category[b]	Bkg[c]	Exposure Levels: Control	Low	Medium	High
Benzenes	ND	ND	•	312±81	907±272
Naphthalenes	ND	ND	*	319:1111	2485±345
Thiophenes and Furans	ND	NO	ND	44±10	2565±187
Phenanthrenes	ND	NO	ND	ND	1208±232
Total Petrogenic Hydrocarbons identified	NO	NO	•	675±1 7S	?47W_l 55

NO = None detected. • = Trace amount detected.

[a] Concentration data reported as mean ±1 standard deviation in ng/g dry weight.

b Each category is composed of the parent compound and alkyl-substituted homologs.

c Bkg = Background tissue sample (tissue sample from organisms collected at the same time as those used for exposure studies).

concentration levels of approximately 2500 ng/g. The total petrogenic hydrocarbon tissue burden averaged 7470 rig/g, clearly the most pronounced bioaccumulation of mussel target tissues examined.

A graphical comparison of petrogenic hydrocarbon tissue levels in testes extracts from the experimental organisms is provided in Figure II-25. This figure presents capillary column gas chromatograms of the aromatic fraction of extracts from each of the four experimental exposure regimes. The chromatograms are divided into five areas which correspond to the hydrocarbon categories used in the previous tabular summaries. As evidenced by Figure 11-25, bioaccumulation of petrogenic hydrocarbons was pronounced at both the medium and high exposure levels.

A summary of petrogenic hydrocarbon bioaccumulation data for each of the target tissues from the adult M. californianus toxicity study is presented in Table 11-91. As evidenced in this table, significant bioaccumulation was limited to gonad tissues and the digestive gland, with relatively low levels detected in the gill tissues. Petrogenic hydrocarbons were not detected in any tissue from background and control animals, and only trace amounts were observed in gonad tissues from the low exposure level. In gill, ovary, and digestive gland tissues, bioaccumulation occurred at roughly comparable levels in organisms exposed to the medium and high level test solutions. Only the testis showed a clear distinction in tissue burdens between animals exposed to the medium and high level test solutions.

Bioaccumulation factors were difficult to calculate for the adult California mussel experiment. Only two compounds, mesitylene and naphthalene, were detected in aqueous test solutions with sufficient frequency to calculate mean concentration levels. Table II-92 presents bioaccumulation factor data for these two compounds in each of the mussel target tissues. These data were generated from the high level exposure study. As evidenced by Table 11-92, neither compound proved particularly appropriate for bioaccumulation factor calculation. Mesitylene was only identified in exposed testis tissues, where it exhibited a bioaccumulation factor of 122. Naphthalene, the other potential compound, was not identified in any of the mussel target tissues and thus could not be used for bioaccumulation factor calculation.

As an alternative to the direct approach, bioaccumulation factors have been estimated for compounds identified in exposed tissue extracts but not detected with sufficient frequency and/or concentration in aqueous test solutions. Table II-93 presents data on estimated bioaccumulation factors for such compounds. The data are categorized into the four groups of parent and alkyl-substituted homologs used for tissue burden presentation. Tissue burden levels and estimated bioaccumulation factors are presented for the high level exposure regime as ranges for the individual compounds identified in each category. Mesitylene and naphthalene have been omitted from the ranges presented in Table II-93. As discussed previously, bioaccumulation factor estimates were calculated using the assumption that the mean aqueous concentration was 0.1 µg/l for each of the individual compounds.

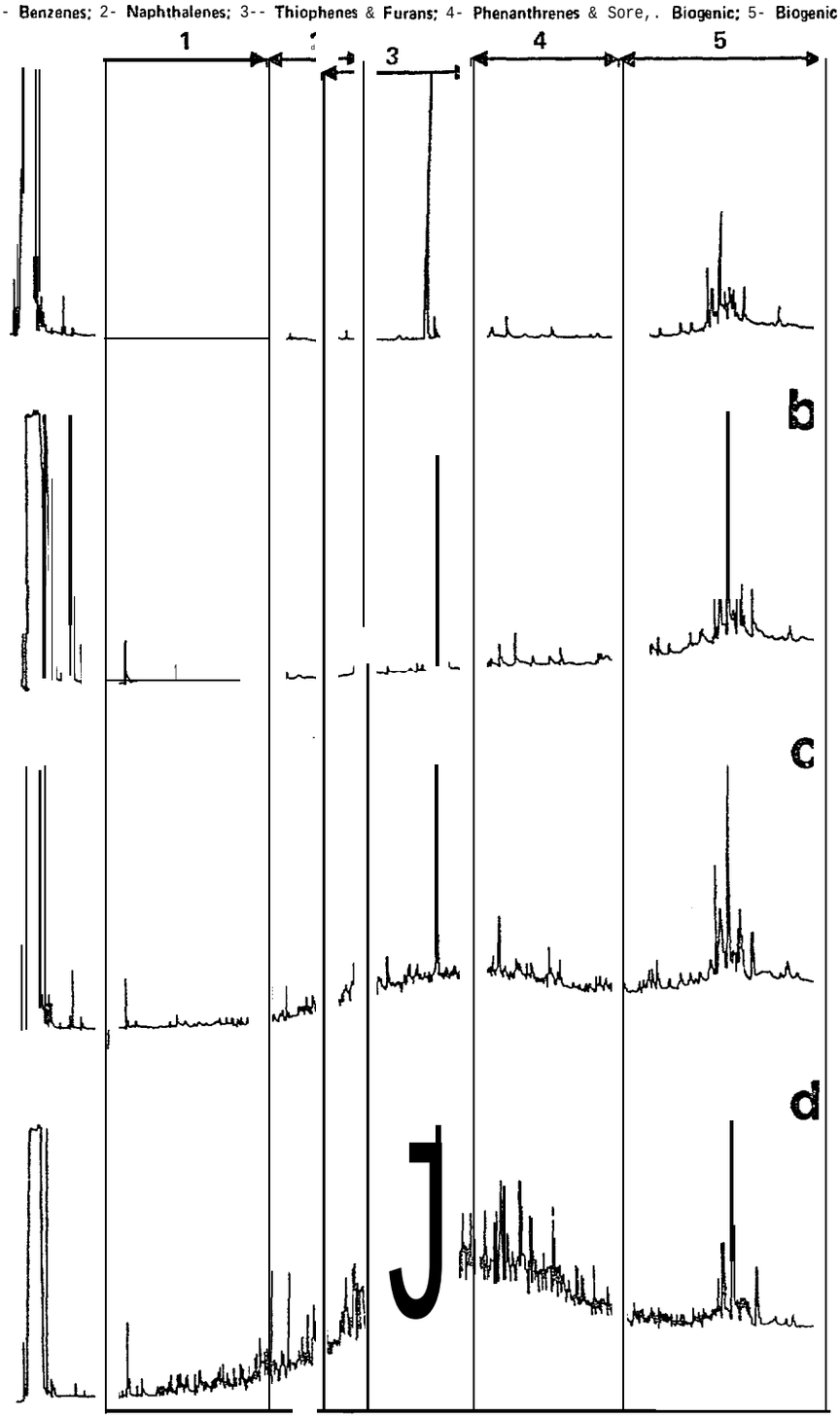


Figure II-25. Capillary column gas chromatograms of aromatic (F2) tissue extracts from California mussel testes: a) control, b) low exposure level, c) medium exposure level, and d) high exposure level.

Bioaccumulation in California target tissues involved the benzene and phenanthrene hydrocarbon families. Mussel target tissues showed low levels of alkyl-substituted benzenes relative to those observed in exposed halibut tissues (Section 2.c.2)). Benzene derivatives were only detected in two of the five mussel target tissues, where estimated bioaccumulation factors ranged from 380 to 2780. Alkyl-substituted naphthalenes exhibited estimated bioaccumulation factors ranging from 380 to 2960 in gonad tissue, and from 550to 1930 in the digestive gland. Thiophene/furan constituents were observed to accumulate in both gonad tissues, with estimated bioaccumulation factors of as great as 10610 in exposed mussel testes. Alkyl-substituted phenanthrenes, which were not detected in any of the halibut tissues, were identifed in four of the five mussel target tissues. Relatively low levels were observed in ovary and gill tissues, but estimated bioaccumulation factors in excess of 4000 were calculated for both digestive gland and testis tissues.

Table II-91. Summary of petrogenic hydrocarbon tissue burden in adult California mussel.

Tissue	Exposure Levels				
	Bkg[b]	Control	Low	Medium	High
Retractor/Adductor Muscle	ND	ND	No	ND	ND
Gill	ND	ND	ND	65±8	77±8
Ovary	No	ND	•	1059±369	815±245
Testis	No	ND	•	675±178	7470±155
Digestive Gland	ND	ND	ND	890±100	1084±92

ND = None detected * = Trace amount detected

[a] Concentration data reported as mean ±1 standard deviation in ng/g dry weight. Values reported are total identifiable petrogenic hydrocarbon tissue burdens.

b 8kg = Background tissue sample (tissue sample from organisms collected at the same time as those used for exposure studies).

3) Survivorship. Adult mussel studies were terminated after 120 days of exposure (i.e. 18 weeks). During the experimental period, daily activities included removal of moribund animals. Survivorship curves through time are displayed on Figure 11-26. Cumulative mortality (Table II-94) on a weekly basis is plotted. As illustrated in the figure, mean survivorship was quite high throughout the entire experimental period. Cumulative mean survivorship ranged from 26.66 to 30.00 individuals/replicate in high exposure regime and control animals, respectively. K-W ANOVA tests performed on cumulative data for two week intervals throughout the entire experimental period revealed no statistically significant differences in survivorship. Similar analyses performed on mortality data for discrete (i.e. non-cumulative) two-week periods during the entire experimental period also revealed no significant differences.

4) Development. All adult mussels were initially marked with identification numbers so they could be individually monitored through the entire

Table 11-92 . Mesitylene and naphthalene bioaccumulation factors for adult California mussel tissues.

Target Tissue	Mesitylene [1,3,5 -trimethylbenzene)			Naphthalene		
	Mean Exposure Level (ug/l)[a]	Mean Tissue Burden (ng/g)[b]	Bioaccumulation Factor	Mean Exposure Level (ug/l)	Mean Tissue Burden (rig/g)	Bioaccumulation Factor
Retractor/ Adductor Muscle	0.45	NO		0.71	ND	
Gill	0.45	ND		0.71	NO	
Ovary	0.45	ND		0.71	ND	
Testis	0 . 4 5	55	122	0.71	ND	
Digestive Gland	0.45	ND		0.71	ND	

NO = Not detected in tissue extracts.

[a] Oata are from the high exposure level test solution.

b Data are from organisms exposed to the high level test solution.

Table II-93. Estimated bioaccumulation factor ranges Of petrogenic hydrocarbons In adult California mussel tissues.

Tissue	Benzenes		Naphthalenes		Thiophenes/Furans		Phenanthrenes	
	Tissue Burden[a]	BF[b]	Tissue Burden[a]	BF[b]	Tissue Burden[a]	BF[b]	Tissue Burden[a]	BF[b]
Muscle	ND		ND		ND		ND	
Gill	ND		ND		ND		77	770
Ovary	196	1960	175-246	1750-2460	53	530	30	300
Testis	38-278	380-2780	38-296	380-2960	104-1061	1040-10610	217-442	2170-4420
Digestive Gland	ND		55-193	550-1930	*		409	4090

NO = Not detected in tissue extracts * = Trace amount detected

a Data represent the range of mean concentrations [rig/g] for individual hydrocarbons identified in tissue extracts from organisms exposed to the high level test solution.

b BF . estimated bioaccumulation factor ranges. assuming a mean aqueous exposure level of 0.1 µg/l for individual hydrocarbons in each category (mesi tyl ene and naphthalene are not included in the ranges),

experiment. At the beginning and termination, of experiments, shell length, height, and girth were measured. Changes in these parameters were calculated and growth was evaluated from this data. A K-W ANOVA was applied separately to data for animals that died during the experiments as well as those that survived the full experimental period. The 'results indicated that no statistically significant differences in growth (i.e. increases in shell length, height, or girth) had occurred during the experimental period in control or any experimental animals (live or dead).

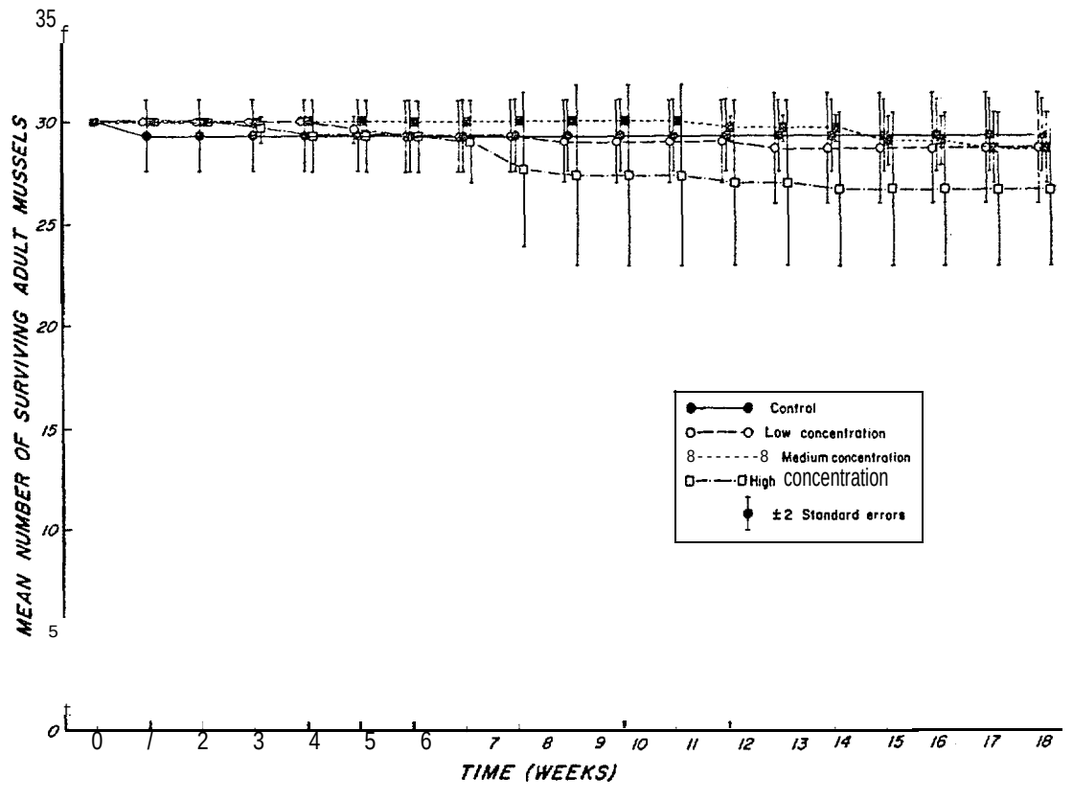


Figure II-26. Mean number of surviving California mussel adults through lime.

Table II-94. Mean number of adult M. californianus surviving per week through 18-week experimental period. Standard errors are in parentheses. A K-W ANOVA revealed no significant differences in cumulative mortality (al biweekly intervals) between control and experimental exposure animals.

Meek	Concentration			
	Control	Lou	Me dium	High
1	29.3 (0.87)	30.0	30.0	30.0
2	29.3 (0.87)	30.0	30.0	30.0
3	29.3 (0. 87)	30.0	30.0	29.67 (0.34
4	29.3 (0.87)	30.0	30.0	29.3 (0.87)
5	29.3 (0.137)	29.3 (0.87)	30.0	29.3 (0.87)
6	29.3 (0.87)	29.3 (0.87)	30.0	29.3 (0.87)
7	29.3 (0.87)	29.3 (0.87)	30.0	29.0 (1.0)
8	29.3 (0.87)	29.3 [0.871	30.0	27.67 (1.871
9	29.3 (0.87)	29.0 (1.0)	30.0	27.33 (2.20)
10	29.3 (0.87)	29.0 (1.0)	30.0	27.33 [2.20)
11	29.3 (0.87)	23.0 (1.0)	30.0	27.33 (2.201
12	29.3 (0.87)	29.0 (1.0)	29.67 [0.341	27.0 (2.00)
13	29.3 (0.87)	28.67 (1.34)	29.67 (0.34)	27.0 (2.00)
14	29.3 (0.07)	28.67 (1.34]	29.67 (0.34)	26.67 (1.871
15	29.3 (0.87)	28.67 (1.34)	29. o (0.58)	26.67 (1.8?)
16	29.3 (0.87)	28.67 (1.34)	29.(J (0.58)	26.67 (1.87)
17	29.3 [0.87!	28.67 (1.34)	28.67 (0.87)	26.67 (1.87)
1s	29.3 (0.87)	28.67 (1.34)	29.67 (0.87)	26.67 (1.87)

Byssus thread production by adult mussels was monitored as an indicator of behavior and physiological function. Weekly counts were made of byssus threads produced by animals in one replicate (30 animals) of each exposure regime (control, low, medi urn, and high). Mean numbers of byssus threads per animal were calculated from these data. Weekly byssus thread production is plotted on Figure II-27. For convenience, statistical comparisons were made on a subset of the total database. Data obtained at two-week time intervals were selected and are presented in Table II-95. Mean byssus thread production per animal ranged from 4.07 threads/animal in the high concentration regime for week 16 to 19.83 threads/animal in the medium concentration regime for week 4. In general, byssus thread production decreased in all experimental animals. Statistical analysis by a K-W ANOVA on biweekly counts revealed that significant differences ($p < 0.05$), with the exception of week 10, existed in byssus thread production for animals exposed to the control and the various test solution exposure regimes (Table II-95}. Multiple comparisons by an analog to the SNK test revealed, with only one exception (week 10), animals in the high concentration exposure regime produced significantly ($p < 0.05$) fewer byssus thl.cads than control, low, or medium exposure regime animals. In some instances, medium exposure animals produced significantly ($p < 0.05$) fewer byssus threads than control and low exposure animals. In addition, on one occasion (week 4) animals in the low concentration produced significantly ($p < 0.05$) more byssus threads than control animals or those from medium and high exposure regimes.

5) Histopathology. Sections of the gut with associated intestine and digestive gland, gill, ovary, testis, and retractor/adductor muscle from 15 animals of each of the three treatment groups (low, medium, and high concentrations) and control were examined.

The gut (Plate II-33b) was lined by tall pseudostratified ciliated columnar epitheliums embedded in loose spongy connective tissue containing tubular elements of the digestive gland. Digestive gland tubules were lined by simple cuboidal secretory epitheliums. Infiltrations of inflammatory cells were regularly observes in the loose spongy connective tissue of the treated groups (e.g. high concentration; Plates II-33b-c), whereas, such infiltrates were not observed in the controls (Plate II-33a). The infiltrates consisted of mixtures of acidophilic granular amoebocytes and macrophages containing brown granules (brown granular cells) (Plate Ii-33a-c). This' was the only significant difference observed between experimental and control groups. All exposed groups (low, medium, and high) showed inflammatory infiltration, with the most severe cases occurring in animals exposed to the high concentration test solution.

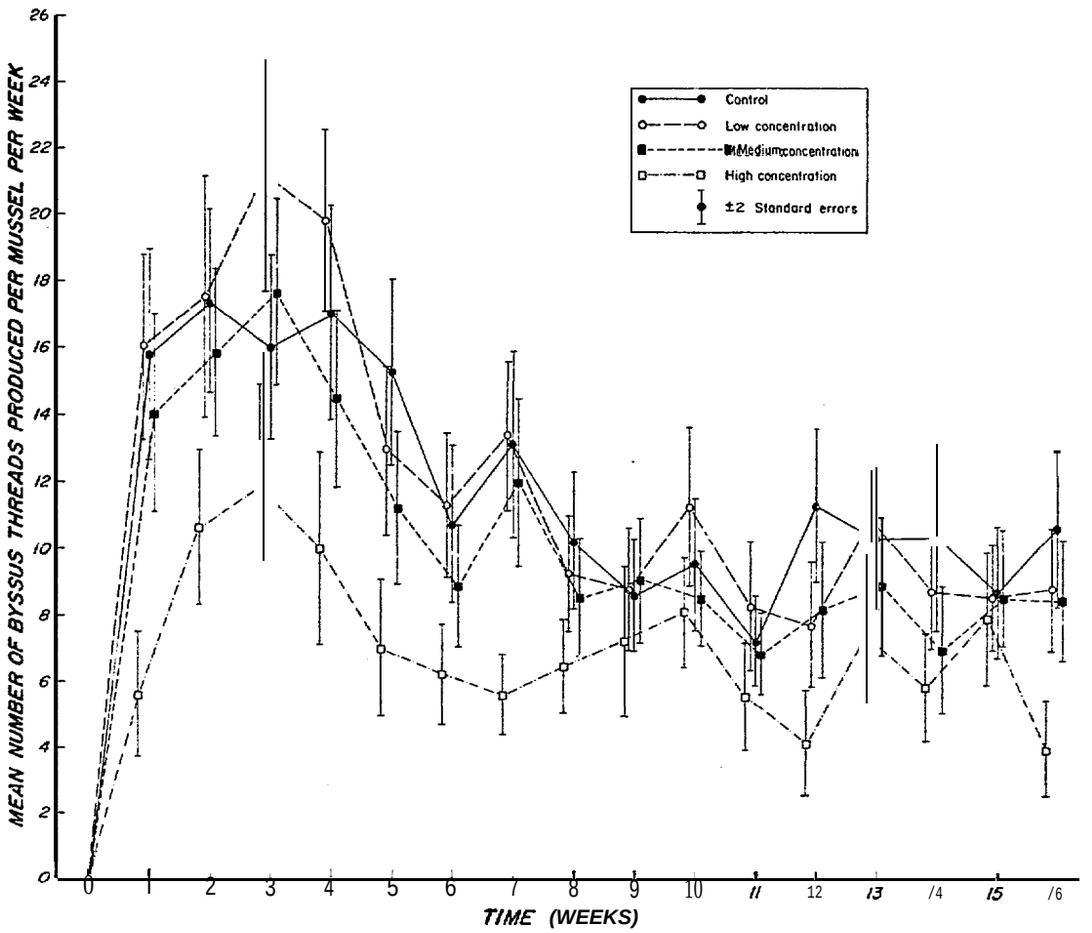


Figure II-27. Mean number of **byssus** threads produced per mussel **per week of experiment.**

Table II-95. Mean byssus thread production (number of threads/animal) by adult M. californianus in control seawaler and experimental test solutions. Statistical results presented for two-week time intervals throughout course of experiments.

	Exposure Period (weeks)							
Concentration	2[a]	4[a]	6a	8[a]	10[a]	12[a]	14[a]	16[a]
Control	16.93[b]	17.07[b]	10.77[b]	10.44"	8.96[b]	11.34[b]	10.97[b]	10.73[b]
Low	18.43[b]	19.83[b]	11.30[b]	9.30[b]	11.30[b]	7.79[c]	*8.83b*	8.88[b]
Medium	15.83[b]	14.47[c]	8.90[b]	8.57[b]	8.86'	8.24[c]	7.04[b]	8.55[b]
Hi gh	9.97[c]	10.03[c]	6.29[c]	6.50[c]	7.69[c]	4.21[d]	5.93[c]	4.07[c]

a Mean number of byssus threads produced per mussel in control and each teSt soluti on Significantly different (p <0.05) among groups; K-W ANOVA.

b,c,d

Byssus thread production groups designated with different letters significantly different [p <0.05) from al 1 other groups; non-parametric analog to SNK mul tiple comparison test. Groups with same letter not significantly different.

Plate II-33(a-c).

a. Representative photograph of M. californianus gut from control animal. Digestive gland (L) with individual tubules indicated by arrows. There are very few amoebocytes in the spongy connective tissue between the tubules compared with Plate II-31b. A portion of testis (T) with numerous mature spermatozoa in two tubules is observed at lower right (X130).

b. Representative photograph of M. californianus intestine (upper right [1]) and digestive gland [L], below and left) from high concentration. Note the presence of numerous acidophilic granular amoebocytes (A) and brown granular cells (macrophages) (B) beneath the epitheliums of the gut and between the hepatic tubules. The elevated numbers of these cells in comparison to the controls was evident (X325).

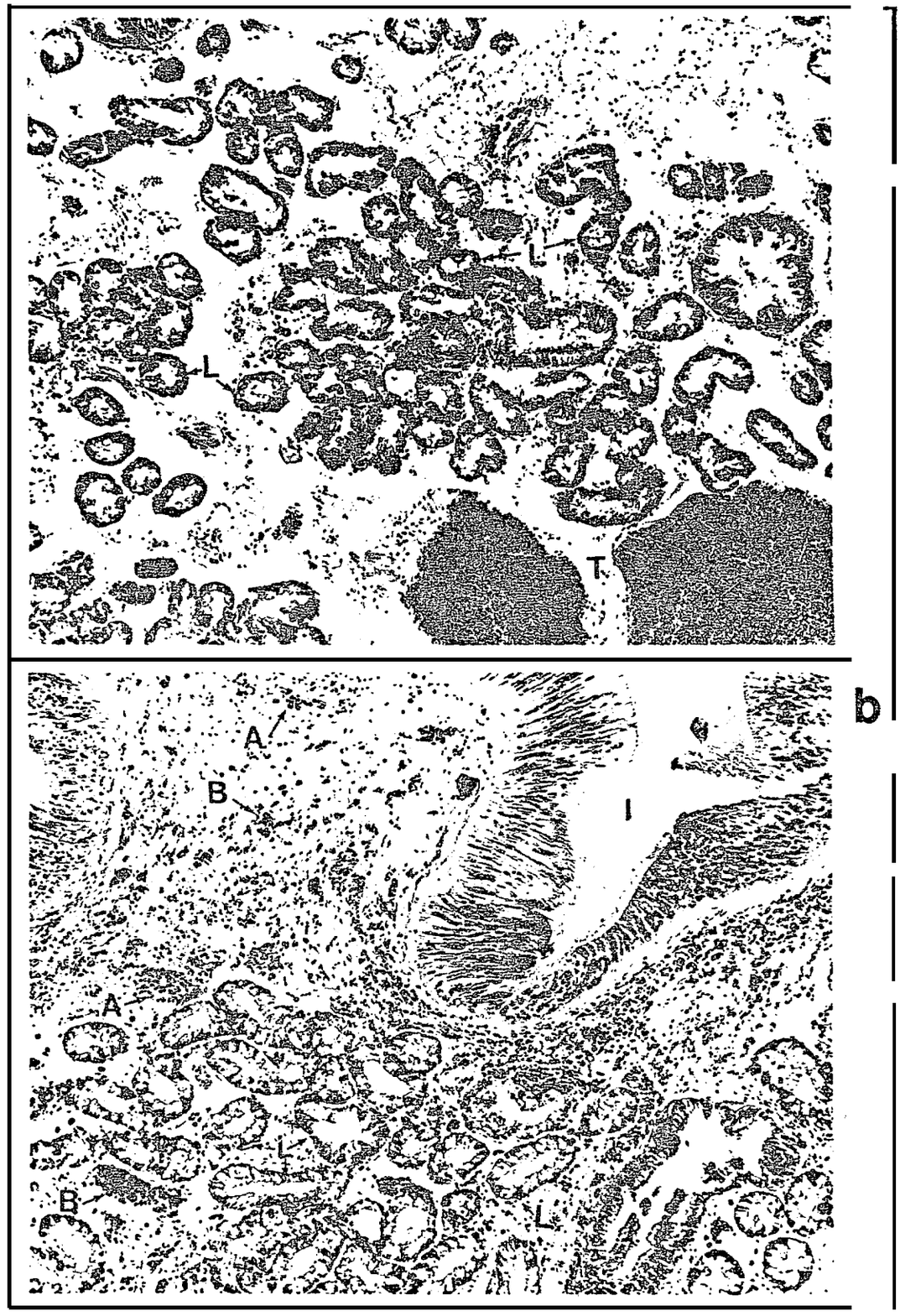

L
L
T
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Plate 11-33 (a-c). (Cent)

c. Representative photograph of M. californianus gut from high concentration treatment regime. Greater magnification (X325) of portion of Plate II-31b. Loose spongy connective tissue in subepithelial locations of the intestine has numerous acidophilic granular amoebocytes (A) and brown granular cells (B), indicative of a non-specific inflammatory response to noxious agents. The staining properties of the two cell types are not observable in this black and white photograph. Acidophilic granular amoebocytes (A) have very small, uniform, brilliantly eosinophilic cytoplasmic granules of variable size. Brown granular cells (B) are macrophages and contain phagosomes.

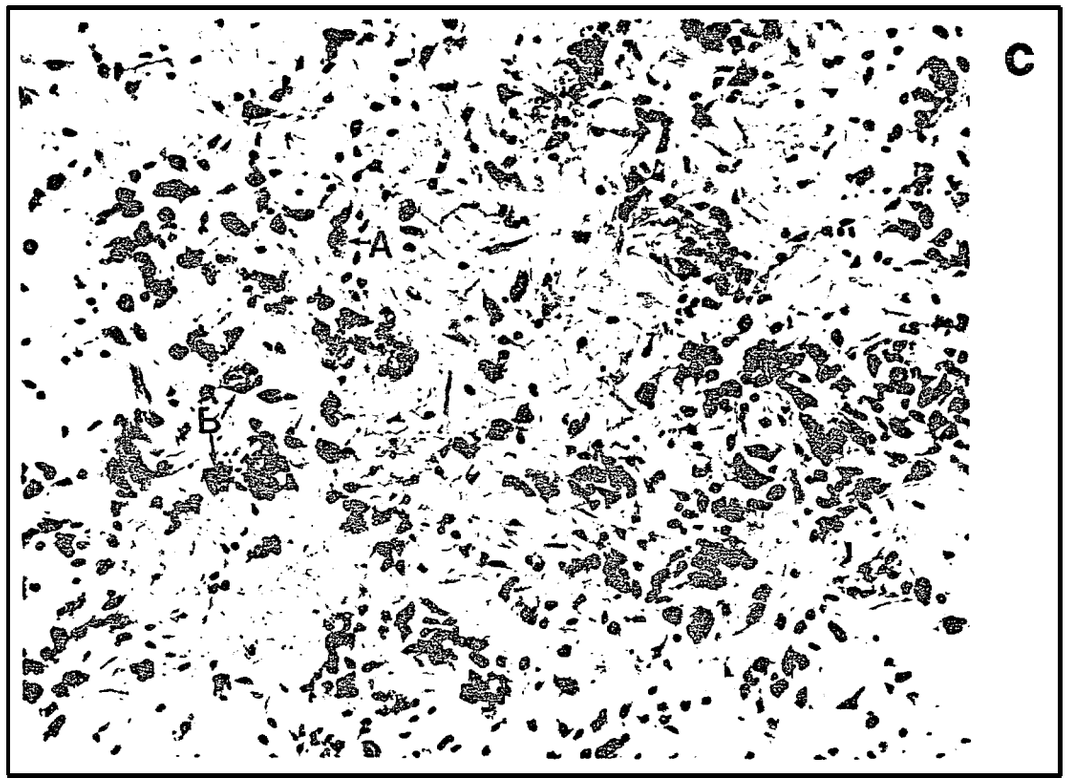

A
B
C

Plate 11-34 (a-b).

a. Representative photograph of M. californianus gill from control animal. The gill appears normal with no significant difference between control and experimental group (X130).

b. Representative photographs of M. californianus gill from high concentration animal. The gill-appears normal and there was no difference between gills of control and experimental groups (X130).

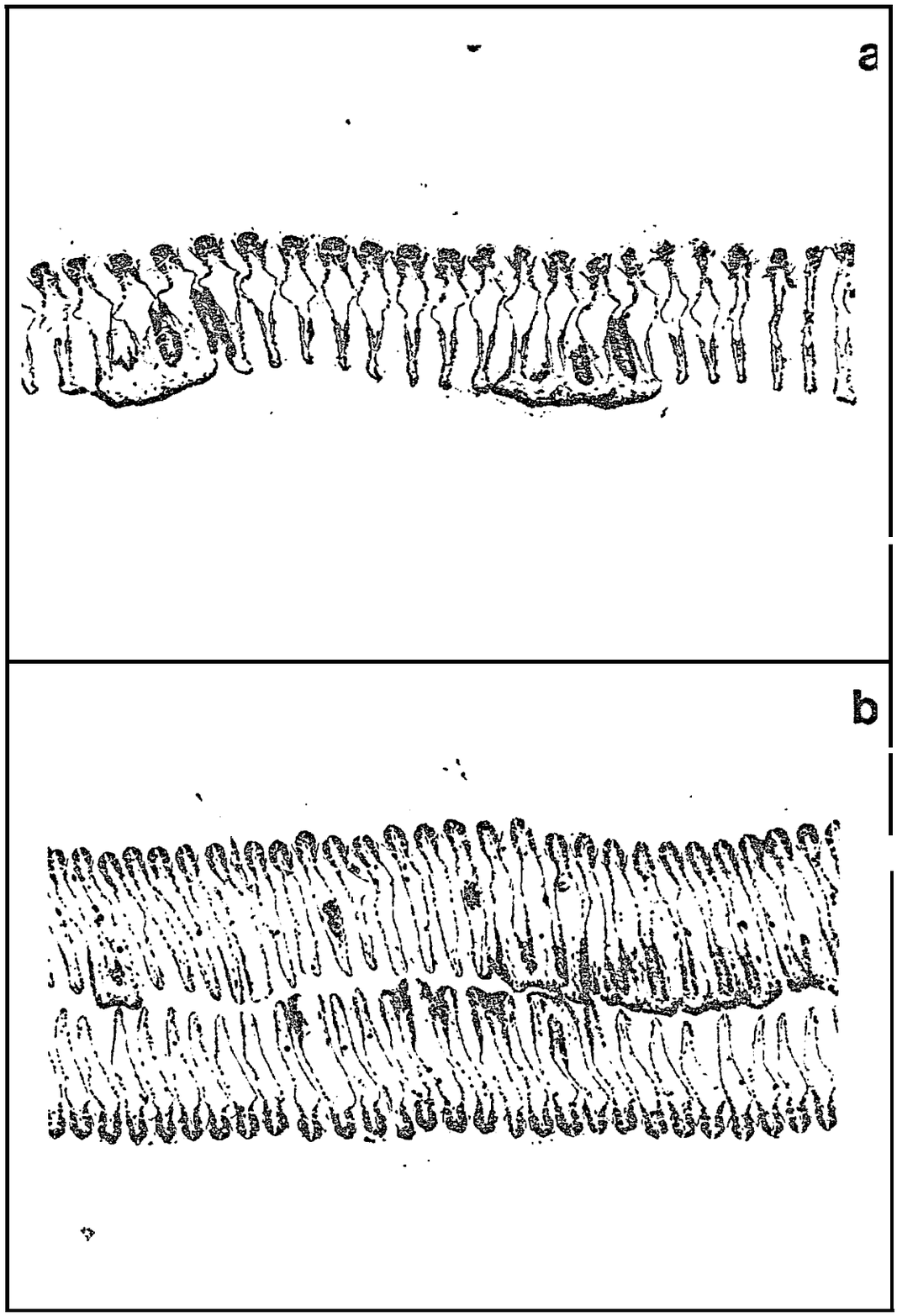

a
b

Plate II-35(a-b).

a. Representative photograph of M. californianus ovary with mature ova (0) from control animal. No significant difference between control and experimental groups (X130).

b. Representative photograph of M. californianus ovary with ova (0) from high concentration animal. No significant difference between control and experimental groups (X130).

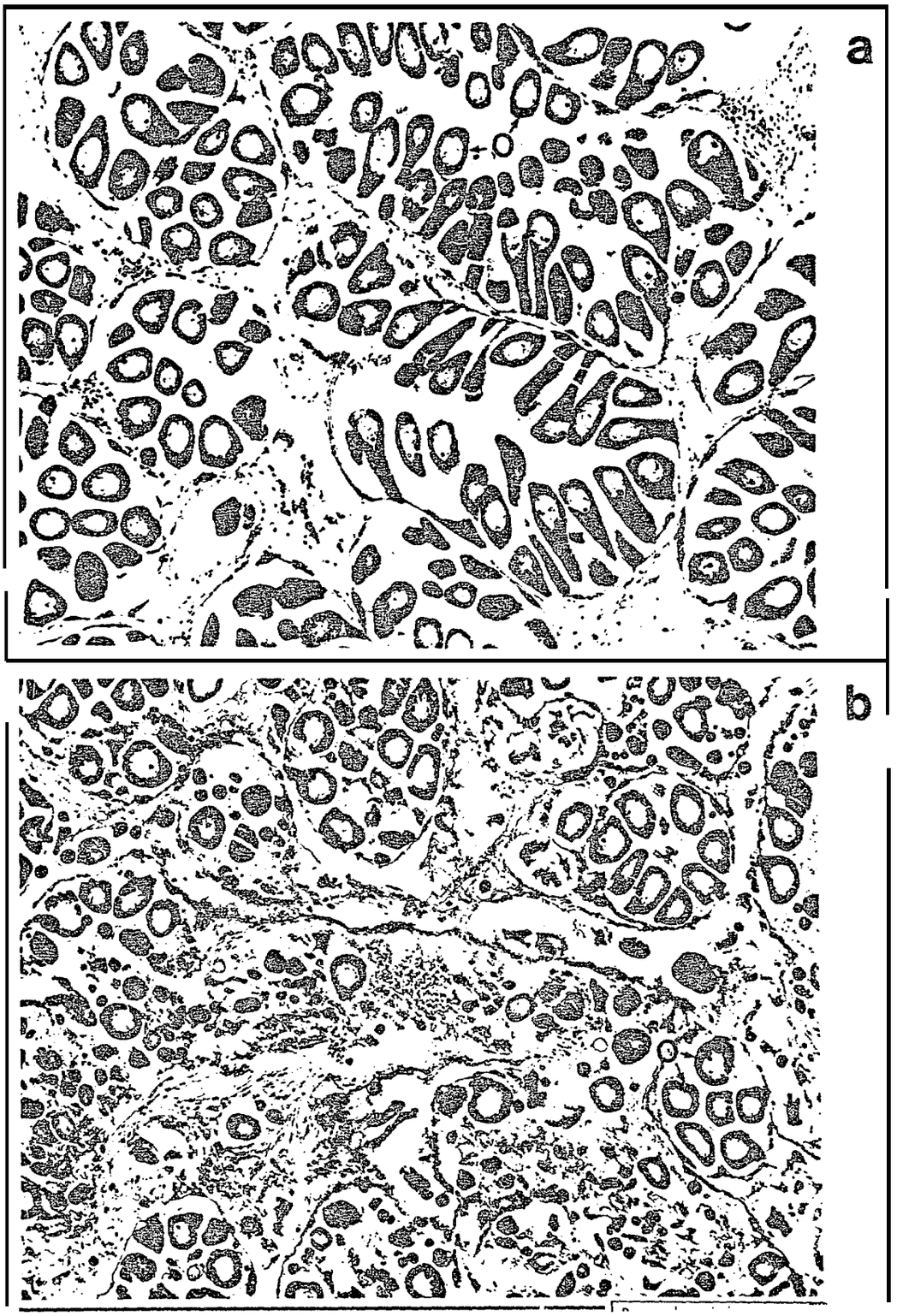
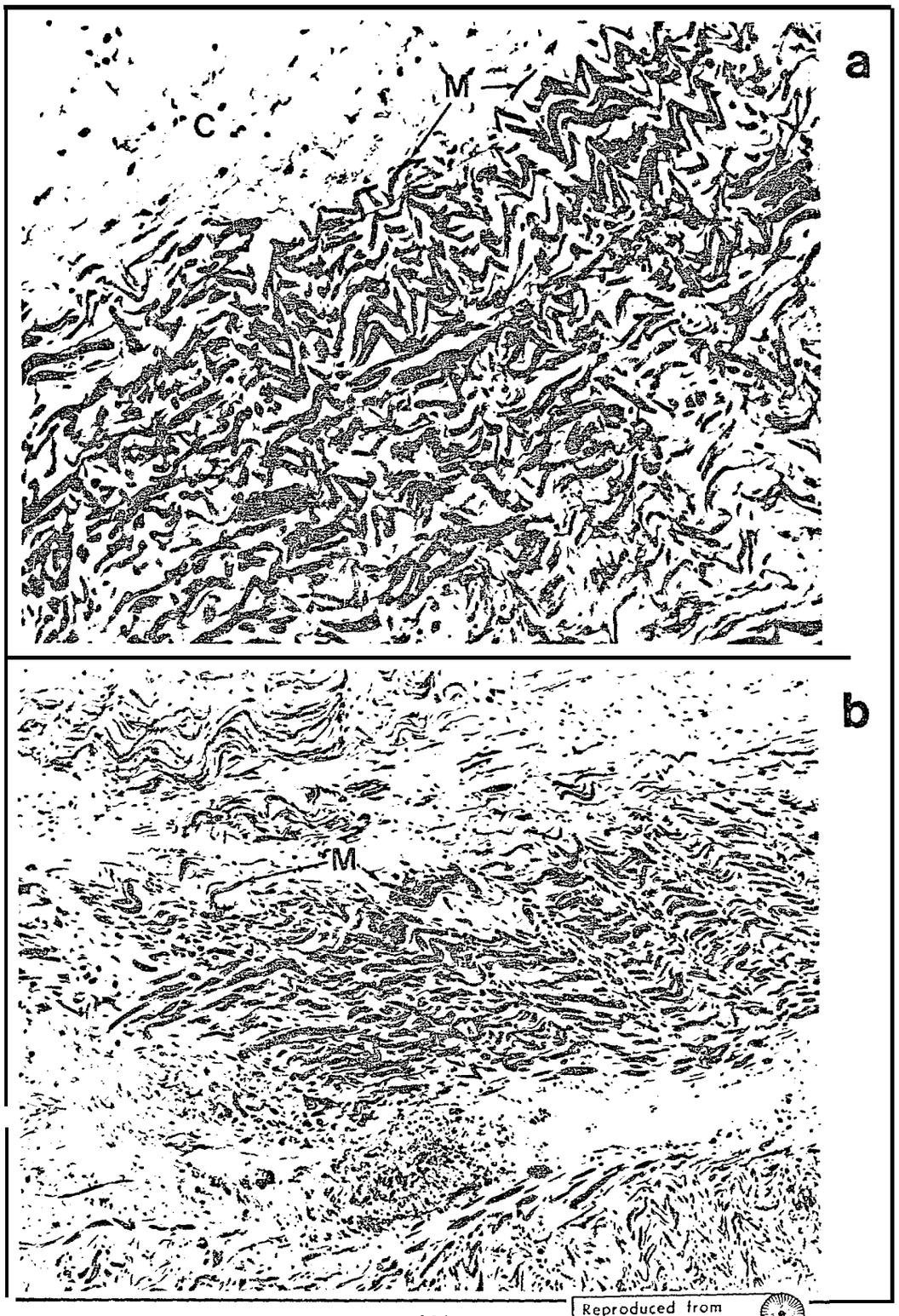

a
b

Plate 11-36 (a-b).

a. Representative photograph of M. californianus muscle cells (M) from control animal. Loose spongy connective tissue (C). No significant difference between control and experimental groups (X325).

b. Representative photograph of M. californianus muscle cells (M) from high concentration animal. No significant difference between control and experimental groups (X130).



Gill tissue consisted of folded plates surfaced by epitheliums. There was no significant difference in the macroscopic appearance of treatment (Plate II-34b) or control (Plate II-34a) groups.

Gonadal tissue, both testes (Plate 11-33a) and ovaries (Plate 11-35a-b], showed mature gametes, but there was no detectable difference between control and exposed specimens.

Muscle tissue from the retractor/adductor muscle of all groups consisted of strap shaped eosinophilic cells without cross stripes. The muscle cells and associated interstitial tissues of the exposed and control groups were not significantly different (Plates 11-36a-b).

6) Behavior. Daily observations were made to assess mortality and any observable changes in appearance or activity. Due to their sessile nature, observations with regard to behavior and activity were somewhat limited. However, of the characteristics which could be evaluated, byssus thread number proved the most consistent and reliable. For this reason, the number of byssus threads produced per animal per week was utilized as the principal measure of behavior and physiologic function (Section 4.c.4)). In addition to byssus thread quantity, other parameters regularly 'evaluated included byssus thread diameter, strength of attachment, response to stimuli (valve closure), integrity of valves (cracks, chips, etc.), mussel grouping, and the relative widths of valve separation during feeding periods (indication of feeding activity).

Throughout the first four weeks of the experiment there were no significant differences in any observable characteristics between control animals and those in the various concentrations. The only mortalities recorded during this period occurred in the high concentration test regime. During this early period, byssus thread counts suggested a trend of fewer threads produced by animals in high concentration exposure regimes.

By the end of week 8, mortality in the high concentration had slightly increased. Many of the mussels which died during this period were observed in a "gaping" posture (valves held 2 to 3 cm apart). The posture was maintained throughout the day prior to death. With the exception of physical damage to the valves, this gaping posture proved to be the best indicator of ensuing death. Weekly byssus thread counts for this period continued to support the already noticeable trend of fewer threads being produced in the higher concentration. Additionally, while counting byssus threads, it was observed that the threads of high concentration mussels appeared to be thinner and somewhat weaker than threads of mussels in other concentrations. A small sample of thread diameters measured from all concentrations supported the observation.

No significant behavioral changes were observed during weeks 9 through 17 of the experiment.

U. DISCUSSION

1. EXPERIMENTAL OIL

Santa Barbara crude oil, the primary toxicant utilized in these studies, proved to be an extremely complex mixture of volatile and non-volatile organic compounds. Results of detailed chemical investigations indicate that Santa Barbara crude is characterized by significant quantities of a diverse array of both aliphatic and aromatic hydrocarbons. This generic composition is typical of mixed-base crude oils produced from marine depositional environments. As such, the chemical composition of Santa Barbara crude is representative of several important classes of crude oils produced and/or transported in marine waters. The compositional similarity is evidenced by both the aliphatic and aromatic hydrocarbon profiles.

The aliphatic fraction of Santa Barbara crude was characterized by a regularly repeating series of n-alkanes and a diverse complement of branched and cyclic hydrocarbons. The normal alkanes were the dominant species in this group of organics, a pattern typical of many crude oils. The distribution pattern and relative abundance of individual n-alkanes in Santa Barbara crude closely reflected that observed for Middle Eastern (Murban) and Alaskan OCS (Cook Inlet and Prudhoe Bay) crudes (Figure 11-10). This distribution pattern sharply contrasts with that of many continental crudes, which generally contain significantly lower levels of n-alkanes and fewer resolved components.

The observed similarities among aromatic component profiles is of even greater importance in the context of the current investigation. Although these compounds were generally present at lower absolute concentration levels in the parental crude relative to aliphatics, aromatic hydrocarbons exhibit a higher aqueous volubility and greater toxicity. The predominant aromatic species identified in the Santa Barbara crude included parent and alkyl-substituted benzenes, naphthalenes, furans, thiophenes, indenes, and phenanthrenes. In general, the C_1 and C_2-substituted species were present at the highest concentrations.

Figure II-28 indicates that the relative abundance of alkyl-substituted benzene species decreases rapidly with increasing substitution, even though the number of potential isomers increases. The relative distribution of naphthalene and its alkyl-substituted homologs in Santa Barbara crude and four other crude oils is depicted graphically in Figure II-29. The prevalence of these compounds in the parental crude oil, coupled with their high aqueous volubility and potential toxicity, makes them a particularly important family of compounds. As was observed for the benzenes, Santa Barbara crude shows the highest relative abundance of C_2-substituted naphthalenes (dimethyl and ethyl derivatives). The distribution pattern is further characterized by an intermediate abundance of methyl naphthalenes (Cl-substituted) and a lesser abundance of the parent compound and C_3-substituted species. This pattern is very similar to that observed for the Middle Eastern and two Alaskan crudes, with all four crudes showing the greatest relative abundance of C -substituted species. Conversely, Long Beach crude exhibits an anomalous a? kyl naphthalene distribution pattern. This crude shows the highest relative abundance of C_3-substituted naphthalenes, with successively decreasing levels toward the parent compound.

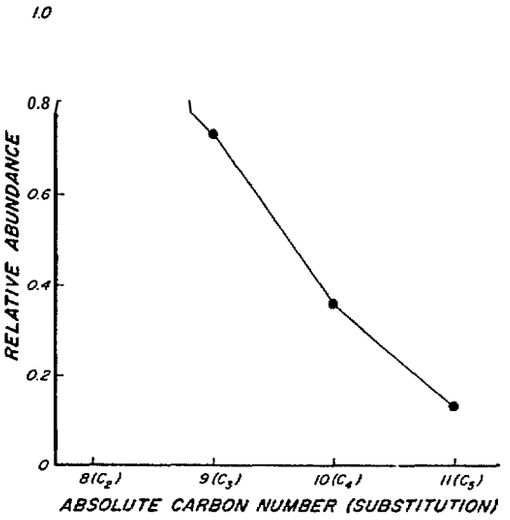


Figure 11-28 . Relative abundance of alkyl-substituted benzenes in Santa Barbara crude oil.

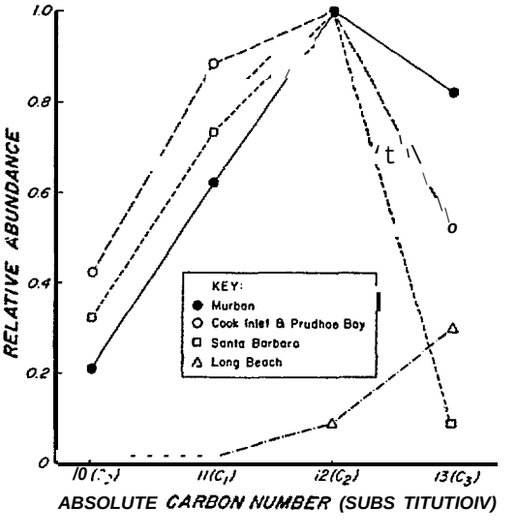


Figure 11-29 . Relative abundance of alkyl-substituted naphthalenes in Santa Barbara and selected other crude oils.

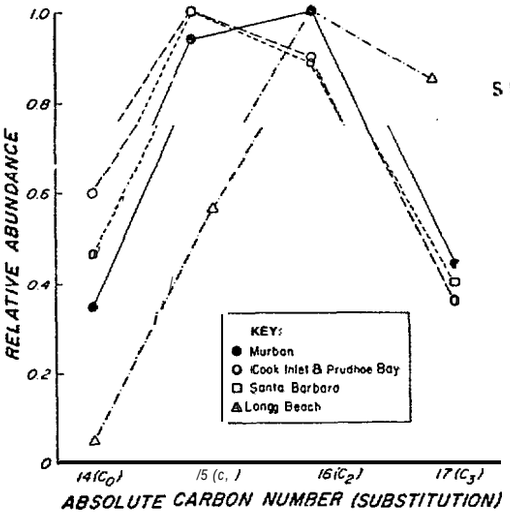


Figure II-30. Relative abundance of alkyl-substituted phenanthrenes in Santa Barbara and selected other crude oils.

Figure 11-30 presents a similar plot for another toxicologically important family of aromatic compounds, phenanthrene and its alkyl-substituted homologs. The distribution data are plotted for Santa Barbara crude and four other crude oils (Prudhoe Bay, Cook Inlet, Murban and Long Beach). Santa Barbara crude exhibits a distribution pattern dominated by methyl -phenanthrenes (C_1 substituted), with a significant contribution from C_2-substituted species and lesser amounts of the parent compound and C_3-substituted phenanthrenes. The relative distribution pattern observed for Santa Barbara crude is again very similar to that of the other oils, particularly the two Alaskan crudes. Although not as pronounced as the naphthlenes, Long Beach crude again has the most unique distribution pattern.

The relative distribution patterns of both alkyl-substituted naphthalenes and phenanthrenes were observed to closely mimic those of Middle Eastern and Alaskan crude oils. These data, coupled with the similarity in aliphatic hydrocarbon profiles, indicate that Santa Barbara crude has a composition representative of many crudes produced throughout the world. This is a particularly important consideration because these observations imply that results of the current toxicity studies should be universally applicable. Careful extrapolation of data obtained from this study to contamination/

spill events involving oil of similar composition thus provides a means of evaluating the biological impact of chronic exposure through utilization of the existing data base.

a. Justification for Hydrocarbon Exposure Levels and Duration

Several considerations contributed to the selection of petrogenic hydrocarbon exposure levels and exposure intervals used in the bioassay experiments. The target exposure levels utilized in the study were 5, 50, and 500 µg/l (parts per billion). The major considerations involved in selection of these target exposure levels included the use of an exposure regime that: 1) was as realistic as possible in terms of observed real-world contamination; 2) included one exposure concentration that would likely elicit an observable toxic effect; and, 3) was based on three target exposure concentrations at order of magnitude intervals so that statistically valid data interpretation would be feasible. Although these considerations are incompatible to some degree, it was felt that all three objectives were important and could be accomplished with the 5, 50, 500 ppb exposure concentration design. Of these three objectives, however, the overriding consideration was to produce an exposure regime that had a sound basis in reality. In this regard, the intention was to utilize exposure levels which had been observed to occur in real-world petroleum contamination events, such as spills, natural seeps, and platform discharges.

In the past 10 to 15 years, a number of research efforts have focused on the nature and extent of petroleum contamination in the marine environment. These research efforts have resulted from intensified exploitation of OCS marine resources, interest in examining potentially adverse effects of such activities, and advances in analytical chemistry which have enabled more accurate quantification of marine petrogenic hydrocarbon levels. Additionally, discrete petroleum spill events in the last 15 years have offered scientists the opportunity to examine the effects of petroleum hydrocarbons on the physical, chemical and biological processes of marine ecosystems.

Although a number of investigations have been focused on quantification of petrogenic hydrocarbon levels in the vicinity of marine oil spills, problems associated with collection and analysis of representative samples have made data correlation and evaluation difficult. Analytical protocols employed in hydrocarbon determinations have involved a wide range of sample extraction/preparation techniques and a variety of analytical techniques. The analytical techniques most commonly utilized include fluorescence spectroscopy, infra-red spectroscopy, thin layer chromatography, gas chromatography and mass spectrometry. These analytical methodologies can vary significantly in terms of sensitivity, accuracy, precision and susceptibility to interference, both among techniques and among individual protocols for a given technique. This problem is further compounded in that variations in sample preparation techniques can also influence the resultant analytical data significantly. Of equal concern are variations in procedures which have been used for sample collection. Because of the relatively low levels of hydrocarbons in marine waters, sample contamination during the collection process is of particular concern.

Despite the problems associated with representative sampling, accurate quantification, and data correlation, several studies have been performed in an attempt to accurately document hydrocarbon levels in the vicinity of petroleum spills. Several of the larger oil spills have been the subject of

detailed scientific investigations in the last 5 to 10 years. Through these studies, we are beginning to develop an understanding of the physical, chemical and biological phenomena occurring in the vicinity of major petroleum pollution events.

Investigations of marine oil spill incidents have focused almost exclusively on examination of non-volatile hydrocarbons, and relatively little work has been done on lower molecular weight volatile species. These lower molecular weight compounds are of particular importance, however, because they include a number of volatile aromatics which are some of the most water-soluble and toxic components of crude oil, Since many studies have not included examination of the relatively soluble volatile components, the hydrocarbon levels observed must be considered conservative estimates of the actual total hydrocarbon levels present.

Table II-96 presents a brief summary of case histories of selected oil spill incidents. The focal point of the summary is centered around a discussion of the water column hydrocarbon levels in the vicinity of the spill. These data have been included to provide some insight on marine hydrocarbon levels which have been observed in the vicinity of real-world petroleum pollution events and thus form the basis for the target exposure levels selected for this study. As the summary indicates, water column hydrocarbon levels in the vicinity of petroleum spills have generally been observed to range from 5 to 400 µg/l, although levels as high as 10,000 µg/l have been reported and a great deal of variation exists. Significant differences in water column hydrocarbon levels have been observed at the same sampling location, as well as among different locations within a given spill area. The variations in resultant water column hydrocarbon levels are a function of the type of material spilled, the mechanism by which it is released, the amount released, the dynamics of the marine system, and the sampling and analytic methods employed.

Table II-96. Summary of oil spill case histories and resulting hydrocarbon concentrations measured in the marine environment.

Spill Event	Location	Volume/ Oil Type	Sample Type	Water Column (µg/l) Concentrations	Reference
Tanker- Arrow	Chedabucto Bay, Nova Scotia	108,000 bbl/ Arabian Crude	Particulate Whole water	16-40 2-131	Levy 1971 Gordon and Michalik 1971
Well-Ekofisk Bravo	North Sea	12,700 tons crude/ 19,000 tons gas	Whole water Whole water	100-400 20-40	Grahl -Nielsen 1978 Audunson 1978
Platform Chevron MPB41C	11 mi east of Mississippi Delta	9,000 tons/ Crude	Dissolved	200	McAuliffe et al, 1975
Refinery Storage Tank	Honshu, Japan	40,000 tons/ Bunker C	Whole water	900-43,700	Anon. 1975
Refinery Storage Tank	Japan	4,000 tons/ Marine Diesel	Whole water	45-60	Hiyama 1979
Tanker- Argo Merchant	Massachusetts	29,000 tons/ #5 Fuel Oil	Whole water	90-340	Jadamec 1978
Tanker-Tsesis	Stockholm, Sweden	17,500 tons #5 Fuel Oil	Whole water	51-58	Kineman et al. 1980
lanker-Kurdistan	Nova Scotia	7,500 tons/ Bunker C	Whole water	0.5-5	Vandermeulen 1980
Tanker-Amoco Cadiz	English Channel	220,000 tons/ Arabian Crude	Whole water Whole water	5-45 26-330	Mackie et al. 1978 Calder et al. 1978
Well-IXTOC 1	Gulf of Mexico	476,009 tons/ Crude	Dissolved Whole water	150-200 100-10,000	Brooks et al. 1981 Fiest and Boehm 1980

[illegal]

[illegal] study was terminated after 84 days. [illegal] of the halibut and anchovy studies, high concen[illegal] terminated earlier because of excessive mortality.

[illegal] the chronic toxicity studies indicate that the criteria [illegal] adequate for selection of sub adult and adult exposure dura[illegal] and were generally satisfied by the protocols employed, [illegal] for embryo and larval studies [illegal] realistic in terms of actual petroleum

contamination incidents. Similarly, the adult exposure regime was intended to elicit sublethal effects from long-term exposure, and as such, fulfilled program objectives. However, because of a paucity of data documenting the temporal profile of water column hydrocarbon levels in the vicinity of real-world petroleum contamination incidents in the marine environment, it is more difficult to objectively evaluate the validity of adult exposure durations.

Virtually no studies have been conducted which have sampled water column hydrocarbon levels in the vicinity of petroleum spills with sufficient frequency to accurately document hydrocarbon exposure profiles. A few spills have been more completely investigated, and samples were collected intermittently over extended time periods. Results of these investigations indicate that petrogenic hydrocarbon levels can persist in the water column at significant concentration levels for up to several months (Table II-97).

As discussed for water column hydrocarbon levels, the rate of change in these levels, and hence the potential duration of exposure, is highly dependent upon the type of petroleum involved, the mechanism by which it is released, the amount initially released, and the dynamics of the marine system contaminated, The magnitude of influence of each factor and the interaction of these variables preclude accurate generalized predictions concerning the level and duration of petrogenic hydrocarbon exposure. Available data suggest that both the exposure levels and exposure intervals selected for use in the current toxicity studies are representative of real-world petroleum contamination incidents.

b. Experimental Test Solutions

Results of chemical characterizations of the WSF test solution generated from the primary crude oil revealed that test solution composition was closely related to the aqueous solubilities of the individual hydrocarbons. Compounds identified with the greatest frequency and at the highest concentration levels were the crude oil constituents of greatest aqueous volubility. With few exceptions, there was generally no direct correlation between the concentration of a given component in the parental crude and its concentration in the WSF test solution. Aliphatic hydrocarbons in general, and n-alkanes in particular, were present at the greatest concentration levels in the Santa Barbara crude; however, because of their limited aqueous volubility they did

Table II-97. Oil spill event duration and persistence of hydrocarbons in the marine environment.

Spill Event	Location	Volume/ Oil Type	Time interval Since Spill	Sample Type/ Concentration (µg/l)	Reference
Tanker- Arrow	Chedabucto Bay, Nova Scotia	108,000 bbl/ Arabian Crude	45 days	Whole water/100	Levy 1971
Well-Ekofisk Bravo	North Sea	12,700 tons Crude/ 19,000 tons Gas	14 days	whole water/20-40	Grahl-Nielsen 1978 Audunson 1978
Refinery Storage Tank	Honshu, Japan	40,000 tons/ Bunker C	90 days	Whole water/200	Hiyama 1979
Refinery Storage Tank	Japan	4,000 tons/ Marine Diesel	23 days 120 days	Whole water /45-60 Whole water/30	Hiyama 1979 Hiyama 1979
Tanker-Tsesis	Stockholm, Sweden	17,500 tons/ #5 Fuel Oil	6 days	Whole water/51-58	Kineman et al. 1980
Tanker-Amoco Cadiz	English Channel	220,000 tons/ Arabian Crude	8 days 8-10 days 45 days	Whole water/5-45 Whole water 26-330 Whole water/4-75	Mackie et al. 1978 Calder et al. 1978 Calder et al. 1978
Well -IXTOC 1	Gulf of Mexico	476,000 tons/ Crude	10-11 mos	Dissolved/150-200 Whole water/100-10,00U	Brooks et al. 1981 Fiest and Boehm 1980.

not contribute greatly to the hydrocarbon content of the test solutions. At the other extreme, polar/heterocyclic compounds were present in the parental crude at very low levels relative to aliphatic and aromatic constituents, yet were major components of the WSF test solution because of their high aqueous solubilities. In this regard, aromatic hydrocarbons occupied an intermediate position relative to the aliphatic and polar/heterocyclic extremes.

Aromatic hydrocarbons, particularly lower molecular weight species, were present at significant levels in both the parental crude and WSF test solutions. This hydrocarbon category showed the greatest degree of correlation between the parental crude and resultant test solution concentration levels. Within this general category, however, there was disparity among individual families of aromatic hydrocarbons. Parent and alkyl-substituted thiophenes, indenes, furans and phenanthenes were typically present at lower concentration levels in NSF test solutions relative to the Santa Barbara crude. Among these compounds the correlation was much stronger than that observed for aliphatic and polar/heterocyclic compounds.

Two examples of the relative distribution of aromatic hydrocarbons between the parental crude and resultant WSF test solution are presented in Figures II-31 and II-32, The strongest correlation was observed for alkyl-substituted benzenes. The relative distribution of alkyl-substituted benzenes in the stock test solution and in the parental crude is presented in Figure 11-31. As evidenced by this figure, the relative distribution pattern was very similar in both media. The distribution patterns were characterized by a maximum abundance of C_2-substituted species which continually declines with increasing substitution. As expected, the test solution distribution "pattern is directly linked to the aqueous volubility of the species involved.

A second example is provided in Figure II-32, which depicts the distribution of naphthalene and its alkyl-substituted homologs in the parental crude and the stock WSF test solution. In the WSF stock solution, naphthalene

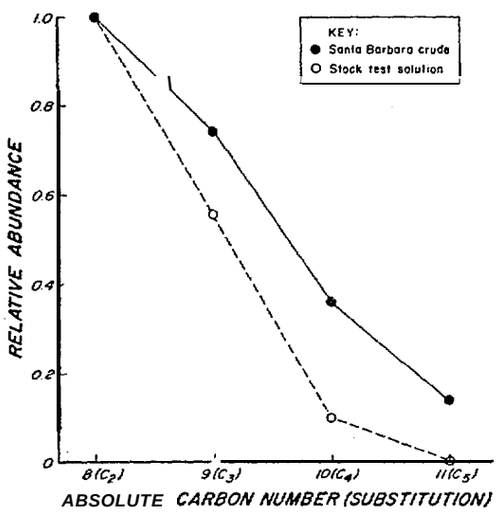


Figure 11-31 . Relative abundance of alkyl-substituted benzenes in Santa Barbara crude oil and the stock test solution.

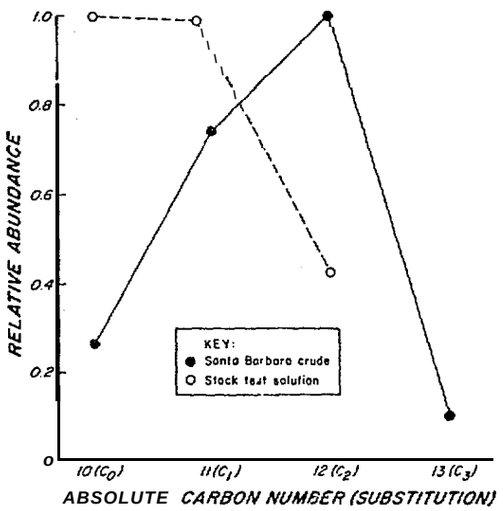


Figure II-32. Relative abundance of alkyl-substituted naphthalenes In Santa Barbara crude oil and the stock test solution.

and methylnaphthalenes (C1-substitution) were present in the greatest relative abundance, whereas C_2-substituted naphthalenes were predominant in the parental crude. The parental crude exhibited a lower relative abundance of naphthalene and methyl naphthalenes, and a pronounced decrease toward C_3-substituted species. Again, the phenomena observed were directly attributable to the aqueous volubility of the compounds involved.

The only general trend observed to occur across all three major hydrocarbon classes (aliphatic, aromatic and polar/heterocyclic) was the dominance of lower molecular weight components in the WSF test solutions. For all hydrocarbon categories, the compounds identified in greatest number and at the highest concentration levels in the WSF test solutions were those in the range of 5 to 10 carbon atoms. Although the parental crude was slightly skewed toward lower molecular weight species (less than 14 carbon atoms) this phenomenon was attributable to aqueous volubility considerations rather than crude oil composition. With very few exceptions, the aqueous volubility of petroleum hydrocarbons decreases rapidly with increasing carbon chain length.

A rigorous monitoring design was employed in this study to accurately define the hydrocarbon exposure profile for each experimental system. Because of the complexity of the dosing apparatus and duration of the exposure studies, it was anticipated that careful monitoring of concentrations would be essential. As initially anticipated, hydrocarbon concentrations were found to be variable over the course of both larval and adult experiments and deviated somewhat from target exposure levels. Hydrocarbon exposure concentrations (corrected for background hydrocarbon contribution) deviated from target exposure levels by an average of 48%, although deviations of as little as 6% were observed and deviations on longer duration adult studies averaged only 22.5%. Similarly, the mean coefficient of variation for hydrocarbon concentration levels in the control and WSF concentration regimes was roughly 51% in larval and adult studies. These deviations and associated variability were the result of several key factors inherent in a study of the type undertaken.

Perhaps the most important factor influencing exposure variability was the inherent complexity of producing a continuous supply of water-soluble petroleum constituents in a flow-through experimental system. As described previously, production of an adequate supply of test solution involved continuous introduction of crude oil and seawater into a system capable of solubilizing the crude oil constituents, separating the dissolved fraction from the dispersed fraction, and removing the "spent" crude oil. This procedure required physically complex apparatus with numerous mechanical components. This entire experimental system was required to generate the primary WSF toxicant, which subsequently was diluted to achieve the desired hydrocarbon exposure concentrations. The reproducibility of the crude oil solubilization process is susceptible to perturbation by external factors such as air temperature, water temperature, and salinity. The combination of these internal and external factors results in a very complex dosing system which was extremely difficult to control with precision over long time periods.

Also critical to dosing system performance, and thus exposure concentration variability, was the duration of the experiments. Chronic experimentation with adult organisms entailed exposure periods of up to 120 days. Some degree of hydrocarbon concentration variability was inevitable when simulating low to intermediate part-per-billion level exposure regimes for experimental periods of this duration. Such variability is particularly difficult to control with a flow-through experimental design requiring continuous generation of petroleum-dosed seawater.

A final factor contributing to the variability of hydrocarbon concentrations and deviations from target exposure values was the fluctuation in the background level of hydrocarbons in the seawater used for the control and three exposure regimes. Some of the variability in background hydrocarbon levels was the result of changes in the composition of the ambient seawater itself, and some was due to fluctuations in the level of diethyltetrahydrofuran, a bioassay system contaminant. Because of the long duration of adult experiments, seasonal changes in ambient seawater hydrocarbon levels appear to have been responsible for some of the variability observed. Although incoming seawater used in these studies was filtered through adsorbent carbon prior to use, a variety of organic compounds are only marginally amenable to removal by such treatment techniques.

Diethyl tetrahydrofuran was consistently identified in the control and WSF exposure regimes during both larval and adult experiments. Additionally, this contaminant was measured at relatively consistent levels in both the control and WSF exposure regimes. This consistency, coupled with analyses of incoming seawater which revealed no detectable levels of diethyltetrahydrofuran, indicate that the diethyltetrahydrofuran was a contaminant introduced from the bioassay system. The bioassay system contained a number of polyvinylchloride (PVC) plumbing components which were glued at appropriate junctures. It is the PVC glue which appears to be the source of the diethyltetrahydrofuran contaminant, as it was used throughout the bioassay system and contains furan derivatives. The presence of diethyltetrahydrofuran is not unusual, although its continual leaching over the two-year experimental period was unexpected.

In larval studies the mean diethyltetrahydrofuran concentration in aqueous exposure media was 7.0+4.0 µg/l, and in adult studies the mean concentration was 8.3+3.7 µg/l. As evidenced by these data, diethyltetrahydrofuran often contributed significantly to the total hydrocarbon concentration in the control and low concentration regime. As a result, the variability in diethyltetrahydrofuran levels nad a direct effect on the variability of total hydrocarbon levels. Its influence was also directly related to deviations from target exposure levels. For these reasons, hydrocarbon exposure data were reported both with and without the inclusion of diethyltetrahydrofuran so that the total exposure level could be distinguished from the petroleum dosed exposure level.

Although the presence of diethyltetrahydrofuran was undesirable, its influence on the results of the chronic exposure studies was considered minimal. Potential interpretation problems were mitigated by the fact that all experimental organisms were exposed to approximately equal levels of diethyltetrahydrofuran. Thus control organisms, which served as the frame of reference for the evaluation of biological effects, were also exposed to diethyltetrahydrofuran. This is important as it means the control organisms remained a legitimate frame of reference to evaluate the effects of petroleum hydrocarbon exposure.

In summary, the variability in hydrocarbon exposure levels and deviations from target values were the combined result of the complexity of dosing seawater with solubilized crude petroleum, the exposure duration and design required in this study, and the variability in incoming seawater hydrocarbon levels (both natural and contaminant). Although this variability sometimes serves to complicate data interpretation, variable hydrocarbon exposure levels were characteristic of real-world contamination scenarios.

Once petroleum hydrocarbons are introduced into marine or estuarine waters, the processes of crude solubilization/weathering and dilution result in highly variable exposure regimes. **Thus., the factor most critical to meaningful interpretation of hydrocarbon exposure effects data is not the inherent** variability in exposure levels or deviation from target exposure levels, but that the actual exposure regime is accurately defined and not merely assumed to be correct.

2. FISH EXPERIMENTS

a. Embryo Experiments

Northern anchovy and California halibut embryos were exposed to three WSF exposure regimes of Santa Barbara Crude Oil from just after fertilization to hatching (approximately 48 to 72 hrs). Total petroleum hydrocarbon exposure concentrations ranged from 10.2 to 367 ppb and 23.9 to 761 ppb in the northern anchovy and California halibut experiments, respectively. Background {seawater controls) petroleum hydrocarbon levels were generally similar to the low concentration regime in both experiments.

Based on measurements of radioactive tracer uptake (^{14}C-naphthalene), both northern anchovy and California halibut embryos exhibited marked bioaccumulation of petroleum hydrocarbons. Bioaccumulation by embryos of both species increased with exposure to increasing WSF concentrations, but was significantly elevated over controls only in the medium (69.5 to 85 ppb) and high (367 to 761 ppb) exposure regimes. Accumulation of petroleum hydrocarbons by fish embryos exposed to crude oil and benzene, respectively, has been reported by Sharp et al. (1979) and Eldridge et al. (1978). Sharp et al. (1979) exposed Fundulus heteroclitus embryos to several dilutions of the WSF of No. 2 Fuel Oil and found that bioaccumulation increased with increasing exposure levels. The 9-day biomagnification factor for petroleum hydrocarbon; was 137 at the highest exposure level. Eldridge et al. (1978) investigated ^{14}C-benzene uptake by Pacific herring embryos and noted that benzene was bioaccumulated in direct proportion to the initial exposure concentration with equilibrium benzene concentrations occurring 6 to 12 hrs following the initial exposure. Herring embryos bioaccumulated benzene to a level 10.9 times that of the initial exposure concentration.

A major difference between the anchovy and halibut results was the magnitude of petroleum hydrocarbon bioaccumulation by embryos exposed to the medium and high concentration regimes. Anchovy embryos exhibited relatively limited bioaccumulation, with medium and high concentration tissue burdens elevated 1.1 and 1.6 times the control level, respectively. In contrast, halibut embryo bioaccumulation in the medium and high concentrations was nearly 30 and 95 times higher than in controls. Differences in embryo bioaccumulation levels at 'the higher exposure concentrations were probably related to one or more of the following factors: 1) a 35% longer exposure period for halibut embryos; 2) higher (approximately 2 times) exposure concentrations for halibut embryos; and 3} the introduction of anchovy embryos to WSF test solutions well after the period of egg capsule water hardening had ended.

Mortality of both northern anchovy and California halibut embryos was found to increase with exposure to increasing petroleum hydrocarbon concentrations. Although anchovy embryos showed a slight increase in mortality at the medium and high exposure concentrations, no statistically significant

difference in mortality could be attributed to WSF exposure. In contrast, halibut embryos exposed to the medium and high concentrations exhibited significantly higher mortality compared to controls. Hatching success of both northern anchovy and California halibut embryos decreased with exposure to increasing WSF concentrations. Hatching success for both species was significantly reduced in the medium and high concentration regimes. No adverse effect on hatching success, however, could be demonstrated in the low (10.2 t023.9ppb) regime.

Elevated embryo mortality 'and reduced hatching success of embryos following petroleum hydrocarbon exposure have often been reported, although most studies have focused on hatching success (Andersen et al. 1977, Sharp et al. 1979, Ernst et al. 1977, Linden 1978, Smith and Cameron 1979 and 1980, Struhsaker et al. 1974). Typically the exposure concentrations reported in the literature are much higher than those used in our experiments with northern anchovy and California halibut embryos. Thus, our results suggest that fish embryos are significantly more sensitive to petroleum hydrocarbon exposure than previously suspected.

Natural mortality rates of northern anchovy and California halibut embryos at sea have not been reported in the literature; however, Dahlberg (1979) indicated that pelagic fish eggs are generally characterized by relatively low survivorship. In his review, Dahlberg (1979) cited embryo survivorship values of 19% to 50% for Black Sea anchovy (Dekhnik 1960, Pavlovskaia 1955), 70% for the Japanese anchovy (Hayasi 1967), 7 to 13% for the Argentinean anchovy (Ciechomski and Capizzoni 1973), and 0.05 to 4.35$ for sole from two locations off England (Riley 1974). Although natural survivorship of pelagic eggs is usually low, it is also highly variable among and within species. Among-species variability is due to differences in reproductive "strategies", whereas within-species variability is due to such site-specific factors as the intensity of predation, water temperature and salinity, water mass transport, and weather conditions (Dahlberg 1979). Based on the results of this study, exposure of northern anchovy and California halibut embryos to chronic petroleum hydrocarbon concentrations of 60 to 80 ppb or more for periods of 48 hrs can be expected to reduce embryo survivorship below natural levels.

The sublethal effects of Santa Barbara Crude WSF exposure on California halibut embryos were assessed by examination of the size (i.e. mean total length} of newly-hatched larvae and incidence of bent notochords. Sublethal effects of exposure on newly-hatched larvae were not examined in the northern anchovy embryo experiment because of differences in the experimental design. Newly-hatched California halibut larvae from embryos exposed to the high (761 ppb) concentration regime were significantly reduced in size compared with control larvae. No reduction in size of newly-hatched larvae was found at the two lower WSF regimes. Similar observations of reduced size in newly-hatched larvae following hydrocarbon exposure have been reported by Leung and Bulkley (1979), Linden (1976, 1978), Struhsaker et al. (1974), and Smith and Cameron (1979) for Oryzias latipes, Baltic herring and Pacific herring, respectively. Struhsaker et al. (1974), exposed northern anchovy embryos to benzene at concentrations of 4.7, 24, and 55 ppm and found significant reductions in the size of developing larvae which were consistent with this study.

Newly-hatched California halibut larvae from all three WSF exposure regimes were characterized by a significantly higher incidence of bent

notochords than were found for control embryos, The incidence of such abnormalities increased with increasing WSF exposure concentrations, reaching a level of nearly 21% in the high concentration exposure regime. Structural abnormalities of larvae hatched from oil exposed embryos have also been reported by Ernst et al. (1977), Smith and Cameron {1979), and Struhsaker et al. (1974). Ernst et al. (1977) found that larvae hatched from Fundulus grandis embryos exposed to the 25% dilution of the No. 2 fuel oil WSF (2.2 ppm TH) had liver, kidney, lens and epithelial tissue abnormalities; however, no abnormalities were reported at lower exposure concentrations. Pacific herring embryos exposed to the WSF of Prudhoe Bay crude oil (PBCO) at 1 ppm for a period of 2 days had a significantly higher frequency of gross morphological abnormalities (primarily bent spines) according to Smith and Cameron (1979). Electronmicrograph (EM) observations revealed that mouths and pectoral fins of herring embryos were also malformed. In subsequent experiments, Cameron and Smith (1980) exposed Pacific herring embryos to a lower concentration (0.7 ppm or 700 ppb)) of the WSF of PBCO for periods of 4 to 144 hours. Although no gross abnormalities were found in newly-hatched larvae, EM observations revealed that muscle tissue mitochrondria were abnormal and that both muscle and brain tissue had large numbers of intracellular spaces. The authors suggested that changes in mitochondrial structure and function may have affected respiration and metabolic rates, resulting in both glycogen and lipid depletion. Struhsaker et al. (1974) exposed Pacific herring and northern anchovy embryos to benzene concentrations of 35 and 45 ppm, and found the frequency of embryonic abnormalities increased with exposure to increasing benzene concentrations.

The reduced larval size at hatching and abnormalities of the body configuration observed in our experiments can be expected to greatly impair early larval swimming performance. Observations of newly-hatched anchovy and halibut larvae indicated that swimming performance of these larvae was in fact impaired. Such impairment will increase the susceptibility of larvae to predation, decrease the ability of larvae to capture prey and grow normally, and generally reduce larval fitness. Together, the effects of exposure to petroleum hydrocarbons during embryonic development can be expected to incrementally reduce ultimate larval survivorship, decrease the overall reproductive potential of the affected species, and possibly contribute to fluctuations in year-class strength if the impact is geographically widespread.

b. Larval Experiments

Northern anchovy anti California halibut larvae were exposed to three WSF exposure regimes of Santa Barbara Crude oil in separate experiments for up to 18 days. Total petroleum hydrocarbon exposure concentrations ranged from 14.4 (low) to 384 ppb (high) and from 10.5 (low) to 606 ppb (high) in the anchovy and halibut experiments, respectively. Background (seawater controls) petroleum hydrocarbon levels were generally comparable in magnitude to the low concentration regime in both experiments.

Radioactive tracer (^{14}C-naphthalene) was added to each !4SF exposure regime and monitored at periodic intervals during each experiment to indirectly assess larval bioaccumulation of petroleum hydrocarbons. Based on such measurements, northern anchovy and California halibut larvae exhibited significant petroleum hydrocarbon bioaccumulation following periods of WSF exposure ranging from 3 to 18 days. Because of extremely high mortality and differences in the experimental design, significant bioaccumulation by anchovy

larvae was only observed in the medium concentration regime after seven days of exposure. The larval halibut experiment, however, clearly showed that petroleum hydrocarbon bioaccumulation was directly related to both exposure concentration and duration of exposure.

The effect of petroleum hydrocarbon exposure concentration on bioaccumulation by larval halibut was most clearly evident after three days of exposure. After three days of exposure there was no evidence of larval bioaccumulation in the low concentration regime, while significantly elevated petroleum residues were indicated in medium concentration larvae. Larval bioaccumulation levels in the medium concentration were elevated nearly 33 times above levels found in control larvae. A similar pattern was evident in low and medium concentration larvae following seven days of exposure; however, tissue burdens of medium concentration larvae were even further elevated over control levels.

The effect of exposure duration on larval tissue bioaccumulation was observed in both the medium and low concentration exposure regimes. For example, halibut larvae exposed to the medium concentration regime exhibited nearly a 5.5 fold increase in hydrocarbon bioaccumulation between days 3 and 7 of the experiment. A similar but more gradual pattern of bioaccumulation was exhibited by larvae in the low concentration. No demonstrable hydrocarbon bioaccumulation was found in the low concentration regime after 3 and 7 days of exposure, respectively. By days 14 and 18; however, tissue burden levels in halibut larvae from the low concentration had increased to levels that were 2.1 and 11.5 times higher than in control larvae, respectively.

Observations on the larval bioaccumulation pattern in the low concentration regime suggest that a bioaccumulation threshold was reached sometime between days 7 and 14 of the experiment. The absence of any significant bioaccumulation through 7 days of exposure indicated that deputation mechanisms operated sufficiently well to eliminate most petroleum hydrocarbons assimilated by the larvae. At some point after day 7, however, petroleum hydrocarbons taken up by larvae began to bioaccumulate. Bioaccumulation beyond this threshold point continued through the remaining days of exposure (18 days). In the medium concentration regime, the exposure threshold probably occurred before day 3.

No previous studies have been conducted on petroleum hydrocarbon bioaccumulation by larval northern anchovy or California halibut, although Eldridge et al. (1978) exposed Pacific herring larvae to a single dose of ^{14}C-benzene and found that benzene was accumulated in direct proportion to the initial exposure concentration. An equilibrium tissue concentration was achieved after about 6 to 12 hrs. Post-yolk-sac larva. exposed to benzene both through water and live food accumulated benzene initially from water and secondarily from food. Yolk-sac and feeding larvae accumulated up to 6.9 and 3.9 times the initial benzene exposure concentration, respectively.

Radioactive tracer uptake by northern anchovy and California halibut larvae clearly established that exposure to petroleum hydrocarbon concentrations ranging from 10 to 700 ppb for periods of 3 to 18 days resulted in very high levels of tissue bioaccumulation. Both lethal and sublethal effects resulted from WSF exposure and tissue bioaccumulation. These effects included reduced survivorship and growth, as well as abnormal development and behavior.

Larval survivorship in the northern anchovy and California halibut experiments was found to be inversely related to exposure concentration and duration of exposure. Anchovy and halibut larvae survived less than 7 days in the high (384 to 606 ppb) concentration regime and less than 14 days in the medium (69.5 to 85.0 ppb) concentration regime. Although both anchovy and halibut larvae exposed to the low concentration (10.5 to 29.9 ppb) regime survived the entire experiments: period, survivorship was significantly reduced compared with controls atter 14 days of exposure.

Few studies have examined the effects of petroleum hydrocarbon exposure on larval fish survivorship. Those that have been performed indicate that survivorship is reduced. Struhsaker et al. (1974), for example, exposed Pacific herring larvae to benzene concentrations of 3 to 55 ppm for 24 and 40 hours, and found that larval survivorship was reduced by 10% to 70% compared with controls. In a similar experiment with anchovy larvae, Struhsaker et al. (19/4) reported that exposure to benzene concentrations of 40 to 55 ppm for 48 hours during the late embryo and early yolk-sac larval stages resulted in significantly reduced survivorship. Our studies clearly indicated that exposure to significantly lower petroleum hydrocarbon concentrations also resulted in heavy mortality. These findings indicate larval fish may be more sensitive than previously believed.

The early larval stages (i.e. yolk-sac and early feeding stages) of broadcast-spawning marine fishes such as northern anchovy and California halibut are generally considered to suffer naturally high rates of mortality (Bannister et al. 1974). Although estimates of early larval stage survivorship for northern anchovy and California halibut have not been reported in the literature, Dahlberg (1979) cited yolk-sac larval survivorship values for other species ranging from 2.3% to 68.0%. Survivorship rates for later larval stages were comparable, ranging from 1.0% to 31.9% (Dahlberg 1979). It is generally accepted that variation in the mortality rate of early larval stages is the principal cause of annual fluctuations in year-class strength. The foundation for this generality is the critical period concept originally proposed by Hjort (1914). Variation in larval survivorship is due to such factors as predation, food availability and feeding success of early feeding larvae, temperature and salinity, water mass movements, and weather conditions (Dahlberg 1979). The results of our oil exposure experiments suggest that northern anchovy and California halibut larvae exposed to petroleum hydrocarbon concentrations of approximately 60 to 80 ppb for periods of 7 or more days will probably suffer additional mortality beyond natural levels. Under conditions when natural survivorship is particularly low and the geographic area of petroleum hydrocarbon contamination is widespread, such additional mortality may have considerable influence on eventual year-class strength.

The sublethal effects of hydrocarbon exposure and tissue bioaccumulation on the growth, development and behavior of northern anchovy and California halibut larvae were examined at periodic intervals during each experiment. In general, the severity of sublethal effects due to exposure and bioaccumulation was related to both exposure concentration and duration of exposure.

The sublethal effects of high concentration (606 ppb) exposure were first observed in the larval halibut experiment after 3 days. Three-day old halibut. larvae in the high concentration regime were both smaller and less well-developed than larvae in the control and other exposure regimes. In particular, the head, posterior gut (i.e. vent), eye, and otic vesicles were either less well-developed or smaller. In addition, the yolk-sac was

significantly larger suggesting a reduced metabolic rate. Larvae were not examined in the anchovy experiment until day 7, thus the effects of shorter exposure periods to lower concentrations could not be assessed.

Northern anchovy larvae exposed to the medium concentration (85.0 ppb) regime for 7 days exhibited significantly reduced growth and were poorly developed. Structural abnormalities included poorly developed jaws, reduced cranial development and ossification, and reduced pectoral fin development. Daily observations prior to day 7 also indicated that post yolk-sac larvae in the medium concentration failed to exhibit normal swimming behavior and did not feed. California halibut larvae exposed to a medium concentration regime of 69.5 ppb for 7 days were also found to grow more slowly, have a higher incidence of structural abnormalities and exhibit abnormal swimming and feeding behavior. Structural abnormalities of halibut larvae included poorly developed jaws and a poorly developed head. No detectable adverse effect of exposure to the low concentration regime (10.9 to 29.9 ppb) was found for either anchovy or halibut larvae following 7 days of exposure.

All northern anchovy and California halibut larvae in the medium and high concentration regimes died prior to 14 days of test solution exposure. Although anchovy and halibut larvae continued to survive in the low concentration regime (10.9 to 29.9 ppb) beyond 14 days, growth of both species was significantly reduced. Northern anchovy larvae were slightly less well-developed than controls, whereas halibut larvae exhibited no evidence of delayed or abnormal development.

It is important to note that reduced growth of northern anchovy and California halibut larvae in the medium concentration regime after 7 days was closely associated with retarded development, abnormal swimming behavior, and the failure to feed. The close correlation between these observations strongly suggests that a combination of reduced development and abnormal feeding behavior resulted in the retarded growth rate. Since all medium concentration larvae in both experiments died soon after yolk-sac absorption, it seems probable that impaired feeding was the primary cause. Although obvious developmental abnormalities and impaired feeding were not observed after 14 or 18 days of exposure in the low concentration regime, both halibut and anchovy larvae exhibited reduced growth. Since mortality was also significantly higher after 14 and 18 days in the low concentration regime, it seems likely that continued WSF exposure would result in complete larval mortality as was observed in the medium concentration regime.

The sublethal effects of crude oil exposure on fish larvae have been investigated by several authors, including Struhsaker et al. (1974), Linden (1975), Sharp et al. (1979), and Eldridge et al. (1977). These studies have consistently demonstrated that petroleum hydrocarbon exposure adversely affects growth, development and behavior of larval fishes. Struhsaker et al. (1974) noted that exposure of northern anchovy larvae to benzene concentrations of 35 to 55 ppm delayed yolk absorption and development and resulted in a higher frequency of abnormalities. Growth rates were generally unaffected except at benzene concentrations greater than 40 ppm. Similar experiments with Pacific herring larvae showed that feeding activity, growth rates, and overall development were also reduced. Linden (1975) reported behavioral abnormalities similar to those observed in this study for larval Baltic herring exposed to No. 2 fuel oil. This abnormal behavior included short vigorous swimming periods followed by slow sinking to the aquarium bottom. Eldridge et al. (1977) investigated the effects of single dose (0.1 and 1.0 ppm) benzene

exposure on yolk-sac and feeding (post yolk-sac) larvae of Pacific herring. Feeding larvae (post yolk-sac larvae) exposed to relatively low benzene concentrations (0.4 microliter/liter) exhibited significantly higher growth and assimilation rates.

As noted earlier, variation in the mortality of early life history stages due to both biotic (i.e. predation, food availability) and abiotic (i.e. physical/chemical) factors is generally thought to be the principal cause of year-to-year fluctuations in year-class strength (Cushing 1975). The basis for this idea is the critical period concept of Hjort (1914 and Cushing 1975). The key premise of this concept is that food availability at the time of yolk-sac absorption and first feeding is the must important determinant of larval survivorship. Thus, if food is available and larvae feed, their survivorship will be relatively high. Conversely, if food is either not available or the larvae cannot feed, then survival will be relatively low. In general, year-class strength will be determined by the magnitude of feeding failure and the overall quality of environmental conditions for those larvae that do feed and survive. The results of our study clearly demonstrate that exposure of yolk-sac and early feeding stage northern anchovy and California halibut larvae to petroleum hydrocarbon concentrations of 60 to 80 ppb for a week will cause structural abnormalities that severely interfere with normal feeding and growth, and eventually cause complete mortality. Our findings further indicate that longer term exposure (14 to 18 days) to very low petroleum hydrocarbon concentrations of 10 to 15 ppb results in reduced growth. The causes of reduced growth are unknown, but smaller-larval size will certainly reduce larval swimming performance. Impairment of larval swimming activities can be expected to reduce larval feeding efficiency and the ability of larvae to avoid predators. These experimental findings suggest that petroleum hydrocarbon exposure prior to and during the period of yolk-sac absorption and early feeding may additionally reduce larval survivorship, as well as influence ultimate year-class strength. The magnitude of such an effect will be dependent on the geographic extent of petroleum hydrocarbon contamination and the correlation between the hydrocarbon contamination event and the peak spawning periods.

c. Adults

1) California Halibut

Petrogenic hydrocarbons were not detected in the tissues from either background or control fish utilized in the adult California halibut experiment. These results suggest there was little petroleum-derived contamination of fish used in the experiment, or that deputation mechanisms had effectively removed parent hydrocarbons from previous contaminations. Similarly, the absence of detectable petrogenic hydrocarbons in control halibut tissues indicates that no inadvertent contamination of control organisms occurred during the experiment or that deputation mechanisms were operative or both.

Eye tissue from halibut exposed to the three WSF exposure regimes exhibited little accumulation of petrogenic hydrocarbons. Halibut exposed to the low concentration regime exhibited no bioaccumulation of petrogenic hydrocarbons, and only a trace was observed in organisms exposed to the medium concentration regime. In fish from the high concentration regime, two alkyl naphthalenes were identified at a combined mean concentration of 140 ng/g. However, it should be noted that tissue mass limitations reduced detection limits by a factor of three and precluded replicate analyses.

Petroleum hydrocarbon bioaccumulation in adult gonadal tissues was very similar to that observed for eye tissue. Only trace levels of alkyl-substituted benzenes and naphthalenes were detected in gonad tissue from organisms exposed to the low and medium concentrations. In the high concentration, two methylnaphthalenes and a trimethyl naphthalene accounted for the majority of the observed 517 rig/g tissue burden. Interpretation of bioaccumulation data in gonadal tissues was confounded by tissue mass limitations that precluded replicate analyses and reduced sensitivity relative to other tissue types by as much as a factor of four.

Muscle tissue from WSF-exposed halibut exhibited some degree of petroleum hydrocarbon bioaccumulation at all three exposure levels. Replicate analyses performed on composite tissue samples and on samples from individual organisms generally showed a high degree of correlation. The positive overall correlation, consistent mean tissue burdens, and small standard deviations indicate that composite sampling provided an effective means of obtaining tissue burden data representative of population variability.

Petrogenic hydrocarbons from the parental crude oil were found to bioaccumulate at trace to very low levels "in muscle tissue from fish exposed to the low and medium concentration regimes. Significant bioaccumulation was only found in muscle tissue from high concentration fish with alkyl naphthalenes accumulated to the greatest extent. Although results are inconclusive from the high concentration regime, tissue burden data suggest that petrogenic hydrocarbons were accumulated too rapidly to be completely eliminated by depuration mechanisms. Gross lipid analysis of muscle tissue showed a lipid content of 0.2%, suggesting that excess petrogenic hydrocarbons may have been sequestered in the lipid depot. Such hydrocarbon sequestering mechanisms have been observed to occur in other marine organisms (Stegeman and Teal 1973). The metabolic cost of such sequestering mechanisms remains undefined, as does the effect of hydrocarbon mobilization should fat reserves be required during periods of stress.

The bioaccumulation patterns observed for adult halibut digestive and liver tissues were quite similar. Although petrogenic hydrocarbons were not detected in tissues from organisms exposed to the low and medium concentration regimes, bioaccumulation was extensive in both tissues from fish 'exposed to the high concentration regime. Adult halibut digestive tissue had a mean petrogenic hydrocarbon tissue burden of 1170 rig/g, while liver tissues contained over 5300 rig/g. The predominant components in both tissues were alkyl-substituted benzenes and naphthalenes. The bioaccumulation pattern observed in both digestive and liver tissues suggests the presence of a deputation mechanism that effectively eliminated and/or metabolized parent hydrocarbons taken up in the low and medium concentration regimes, but not in the high concentration regime. Gross lipid analysis revealed that gut tissue lipid content was 1.7%, the highest level of the three halibut tissues (muscle, gut, and gill) examined. The relatively high lipid content, particularly when compared to the muscle tissue, indicates that accumulated hydrocarbons may have been sequestered in the lipid.

Of the adult California halibut target tissues investigated, gill tissue was the most severely impacted in terms of petrogenic hydrocarbon bioaccumulation. Bioaccumulation in gill tissue was significant in all three concentrations, with mean tissue burdens of 63, 145 and 6916 rig/g in the low, medium and high concentrations, respectively. Figure II-33 presents a graphical summary of the composition and total gill tissue burden for fish

from each exposure level. As this figure indicates, alkyl-substituted benzenes and naphthalenes were accumulated to the greatest extent in gill tissue. This trend is consistent with that observed in all of the halibut target tissues, and provides further evidence of the enhanced bioavailability of these compounds relative to other WSF components.

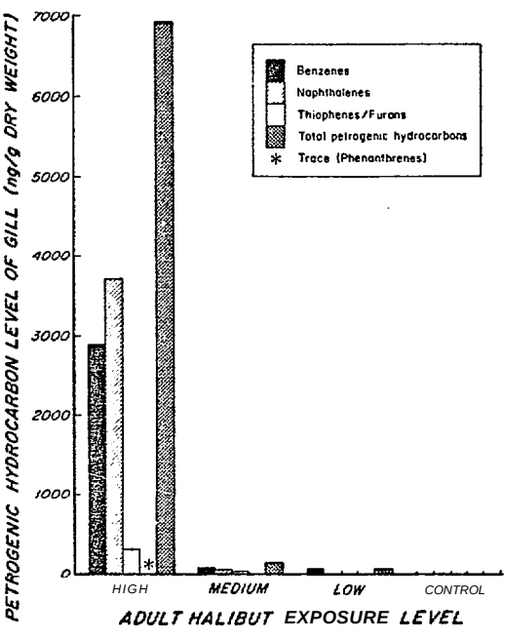


Figure II-33. Petrogenic hydrocarbon burden profile in adult California halibut gill tissue.

Bioaccumulation factors as high as 12000 were estimated for individual aromatic hydrocarbons in halibut gill tissues from the high concentration regime. C_3-substituted derivatives of both benzene and naphthalene exhibited the largest estimated bioaccumulation factors. Estimated bioaccumulation factors generally increased in direct relation to the molecular weight of the analog. This trend was observed to occur in both groups of aromatic hydrocarbons, and is consistent with results from similar studies involving the exposure of clams to the WSF of No. 2 fuel oil (Neff et al. 1976b).

Since petroleum hydrocarbon uptake is largely a function of active biological processes, gill tissue seems to represent a particularly susceptible organ. The continuous activity associated with respiration, and the membranes across which gas exchange occurs, provide an excellent mechanism for hydrocarbon uptake. A possible explanation for the extensive bioaccumulation in halibut gill tissue is the relatively high lipid content of these tissues. Adult halibut gill tissue had a gross lipid content of 1.0%, suggesting that petroleum hydrocarbons may have been sequestered in deposited fat via partitioning processes subsequent to transfer across gill membranes. Unfortunately, definitive evidence of the lipid partitioning process is generally lacking, and virtually no previous data are available on bioaccumulation of petroleum hydrocarbons in California halibut tissues.

Figure II-34 presents a graphical summary of the total petrogenic hydrocarbon tissue burden in each of the adult halibut tissues. With the possible exception of gill tissue, all tissues exhibited a bioaccumulation pattern indicative of a classic threshold effect. Exposure to the low and medium concentration exposure regimes resulted in either non-detectable or very low levels of bioaccumulated hydrocarbons, whereas bioaccumulation was pronounced in tissues from halibut exposed to the high concentration regime. These data indicate that deputation mechanisms were capable of metabolizing and/or eliminating parent petrogenic hydrocarbons from target tissues at the low and medium exposure levels. However, in the high concentration regime, petroleum hydrocarbon uptake apparently occurred too rapidly, or to such a magnitude that depuration mechanisms were overwhelmed and parent hydrocarbons accumulated in all target tissues. In terms of bioaccumulation, it thus

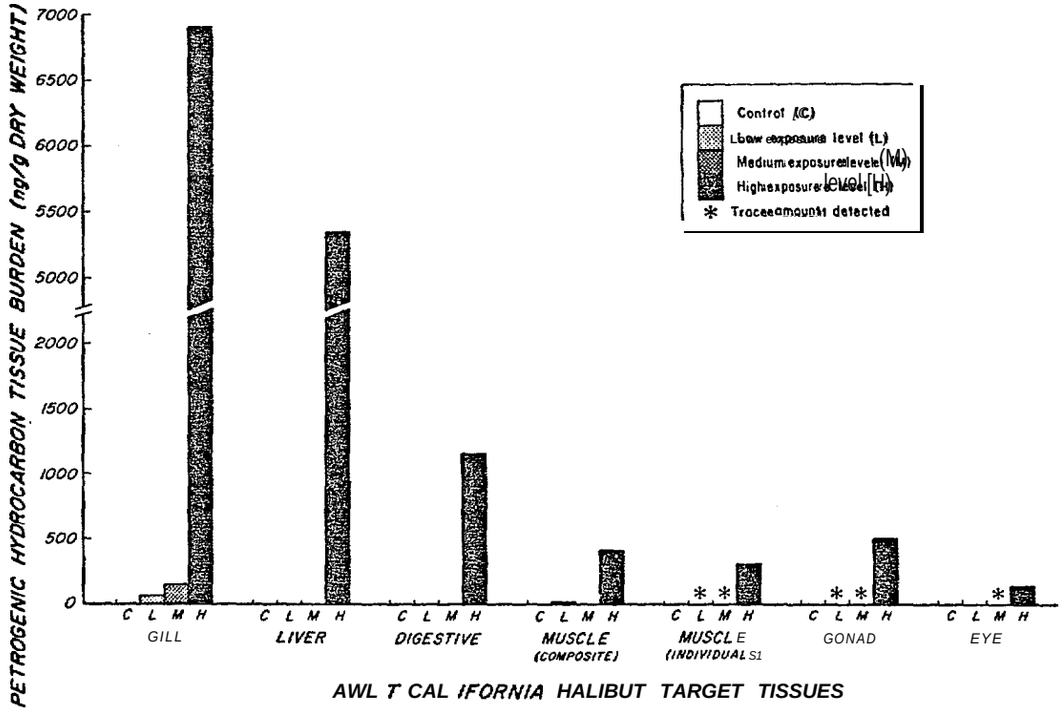


Figure II-34. Total petrogenic hydrocarbon burden in adult California halibut tissue.

appears that a depuration threshold was reached at an exposure level somewhere between the medium (46 ppbs) and high (417 ppb) exposure levels.

Survivorship of adult California halibut exposed to the WSF of Santa Barbara crude oil was related to both the exposure concentration and the duration of exposure. Cumulative survivorship of halibut was inversely proportional to the hydrocarbon exposure concentration with halibut survivorship significantly lower in the high concentration regime (417 ppb) than in either the medium (46.6 ppb) or low (9.5 ppb) regimes. This pattern initially became evident after five weeks of WSF exposure and continued throughout the duration of the experiment (12 weeks). Although cumulative survivorship of halibut was lower in the medium and low concentration regimes than in the controls, no statistically significant difference could be detected.

The effect of exposure duration on halibut survivorship was evident in all three WSF exposure regimes, but was most striking in the high concentration regime. Halibut in the high concentration regime began to die before fish in the other regimes and all fish had died by the eighth week of the experiment. Survivorship of halibut in the medium and low concentration regimes decreased gradually over the 12-week experiment, but initial mortality occurred first and survivorship was always lower in the medium concentration.

Numerous short-term (4 to 5 day) bioassay experiments have been conducted using marine fish (Anderson et al. 1974, Moles et al. 1979, Rice et al. 1977, Hedtke . ... Puglisi 1980, Kern et al. 1979, Moles 1980, Benville and Kern 1977, Meyerhoff 1975) that clearly demonstrate the toxicity of various crude oils and single compound toxicants (e.g. benzene, toluene). Typically,

however, these studies have utilized total hydrocarbon concentrations in the ppm range, levels which greatly exceed even the high concentration regime (417 ppb or 0.417 ppm) used in our study. A relatively small number of longer term (4 months or greater) chronic bioassay studies have been conducted using various petroleum hydrocarbons and marine fishes (Fletcher et al. 1979, McCain et al. 1978, Payne et al. 1978a). Each of these studies reported no or negligible mortality of test fish; however, the test conditions in each case were quite different from our study. Fletcher et al. (1979) and Payne et al. (1978a) utilized crude oil layered on water, but failed to measure actual hydrocarbon exposure concentrations. In contrast, McCain et al. (1978) employed sediments that were experimentally oiled to a concentration of approximately 400 to 700 ppm. Although it is difficult to compare results of the halibut experiment with these studies, they suggest that California halibut are more sensitive to petroleum hydrocarbon exposure than previously suspected.

Growth of adult halibut during WSF exposure was assessed using the percentage weight change rather than actual weight change because of differences in exposure duration for individual fish. Nearly all W.SF-exposed and control fish lost weight during the experiment, although a small percentage of individuals exhibited a slight weight gain. Since neither the mean percentage weight loss per day for fish from all exposed regimes, nor the mean percentage weight loss for those fish surviving to the end of the experiment were significantly different, no adverse effect on growth (or weight gain) can be attributed to WSF exposure.

Although halibut growth was not adversely affected by WSF exposure in this experiment, other studies have demonstrated reduced growth of fish exposed to petroleum hydrocarbons. Juvenile striped bass exposed to sublethal concentrations of benzene (3.5 and 6 ppm microliter/liter) for four weeks were found to have significantly reduced wet and dry body weights and percent body fat (Kern et al. 1976a) when compared with control fish. Benzene exposure also induced hyperactivity and decreased feeding success probably through the impairment of food location. McCain et al. (1978) reported a weight loss for English sole exposed to oil-contaminated sediments (400 to 700 ppm) for periods of up to 4 months.

Reasons for the observed halibut weight loss are not clear, but may be related to several factors. First, behavioral observations indicated many fish did not respond well to food, and that large amounts of food remained in experimental tanks after feeding. Second, both control and WSF-exposed fish were subjected to the additional stress of a parasitic leech infestation. Finally, the experiment was conducted in late winter/early spring when water temperatures were relatively low. Many flatfishes are known to reduce their metabolic rate in winter and some stop feeding completely (Miller 1967). Such a response by halibut to low water temperatures may have contributed to reduced feeding and the observed weight loss of both control and WSF-exposed fish.

Histopathological observations were made on gill, liver, gonad, pancreas, eye and skeletal muscle tissues from halibut exposed to the medium, low and control regimes. Because of rapid death and subsequent tissue autolysis, reliable observations could not be made on fish from the high concentration. Significant effects due to WSF exposure were observed only in gill and liver tissues. All other tissues showed no significant difference between experimental and control regimes.

Gill pathology was nonspecific, but included evidence of significant damage such as hyperplasia of epitheliums, inflammatory infiltration and fibrosis of secondary lamellae, fusion of the tips of secondary lamellae, and epithelial cyst formation. Microaneurisms were also observed at the tips of secondary lamellae. Any or all of these changes have been observed as a result of injury by a variety of chemical, physical, and biological agents (Wellings, unpublished). In the present instance the observed gill damage was attributable to WSF-exposure. Such changes have been repeatedly observed in fish and documented in publications reviewing the effects of a variety of organic compounds on fish tissue structure (Ribelin and Migaki 1975, Roberts 1978).

Gill changes, including secondary lamellar hyperplastic thickening, were observed by Cech (1983) following exposures of Ictalurus punctatus to chloramine. In studies of Morone saxatilis mild pathological changes occurred in gills of fish experimentally exposed to benzene, zinc, and monochloramine. These changes sometimes included thickening of secondary lamellae and increase in numbers of mucous cells. In contrast, Payne et al. (1978a) reported no histopathological changes in gill, liver, kidney, heart, gonad, spleen, or muscle of cunners (Tautogolabrus adspersus) chronically exposed to Venezuelan crude oil.

The liver histopathology observed in the present experiments consisted most significantly of individual cell necrosis and increase in size and numbers of melanin microphage centers. There was also glycogen depletion and a tendency toward fat vacuolation of hepatocytes. Both conditions are considered non-specific and unrelated to any particular chemical or physical agent. Similar changes have been observed in fish (Whipple et al. 1978) and in other classes of vertebrates by numerous authors (see Ruebner and Montgomery 1982, Thomson 1978, Anderson and Kissane 1977). Specifically dealing with flatfish, the review of Hodgins et al. (1977) and the work of McCain et al. (1978) detail similar hepatic and other pathology.

In the present experiments many of the changes observed by others in several organ systems, including gill and liver, were not found. Moreover, changes observed were generally less severe than those noted by other workers, who generally used higher exposure levels and different experimental conditions. Unfortunately, the vast bulk of the pertinent toxicological literature includes no histopathological data. Finally, there was no evidence of pre-neoplastic or neoplastic conditions that have previously been observed in fish (Schlumberger and Lucke 1948, Wellings 1969, Mawdesley-Thomas 1975).

2) Northern Anchovy

Petrogenic hydrocarbons were not identified in either of the two target tissue groups (muscle and head/viscera) from background northern anchovy. Replicate target tissue analyses were performed on samples composited from randomly selected individuals within the pool of organisms utilized for the WSF exposure studies. These data indicate that experimental organisms were not contaminated with petroleum-derived hydrocarbons at the initiation of the experiment. Replicate analyses of target tissue groups obtained from control animals sacrificed at 30-day intervals over the 120-day experiment revealed no detectable levels of petrogenic hydrocarbons. The absence of detectable petrogenic hydrocarbons in control tissue groups indicated that control organisms were not inadvertently contaminated during the 120-day experimental period. These observations suggest that animals collected for use in the study were healthy at the initiation of the bioassay, and verify that

that control organisms provide an appropriate frame of reference for evaluation of bioaccumulation in target tissues from WSF-exposed northern anchovy.

Inherent limitations associated with the relatively small size of adult northern anchovy, the sensitivity of the analytical techniques employed for bioaccumulation assays, and the experimental design were the primary factors dictating selection of target tissues. To achieve the desired low part-per-billion detection limits for individual hydrocarbons, 10 to 20-gram tissue masses (wet weight) were required. This requirement, coupled with the size of adult anchovy and the subsampling protocol in the experimental design, precluded examination of individual organs. Although composite sampling techniques were utilized, the numbers of organisms required to obtain the requisite tissue masses were considered prohibitive. These considerations resulted in selection of skeletal muscle and a combined head/viscera group as the two target tissues utilized for bioaccumulation studies. The head/viscera target tissue group was composed of the gill, the skull and its entire contents (e.g. brain and eye), and all of the visceral organs.

The experimental design used in the adult northern anchovy study was altered to obtain a more complete profile of the effects of chronic WSF exposure. The protocol entailed subsampling of fish at 30-day intervals over the course of the study, rather than a single sample at the end of the experiment. Thus, histological examinations and bioaccumulation assays were performed on tissues from fish sacrificed after 30, 60, 90 and 120 days of exposure. This approach facilitated better definition of the exposure duration at which adverse effects were manifested, and provided greater insight into the nature and rate of petroleum hydrocarbon bioaccumulation. All high concentration fish died prior to day 60, thus, only one set (30-day exposure) of tissue samples was available for bioaccumulation evaluation from this regime.

Although the absolute bioaccumulation levels varied considerably between the two adult anchovy target tissues, the general bioaccumulation patterns were very similar in each. Figures II-21 and II-22 provide graphical representations of the bioaccumulation patterns observed during the experiment for the skeletal muscle and head/viscera tissues, respectively. Muscle and head/viscera tissues exhibited consistent and similar relationships between exposure concentration and hydrocarbon tissue burden for each of the four exposure periods. Additionally, the type and relative proportion of accumulated petrogenic hydrocarbons were consistent in both tissues types, as well as within a given tissue type for each exposure period.

After 30 days of WSF exposure, parental petrogenic hydrocarbons had not bioaccumulated to detectable levels in either the skeletal muscle or head/viscera tissue from northern anchovy exposed to either the low and medium concentration regimes. In contrast, bioaccumulation in muscle tissue from anchovy exposed to the high concentration regime was extremely high. The mean muscle tissue burden was 1653 rig/g and head/viscera tissues had accumulated petrogenic hydrocarbons to a level of 6700 rig/g. The composition of the tissue burden was very similar in both target tissues; alkyl -substituted benzenes and naphthalenes represented 38% to 44%, indene derivatives contributed 9% to 18%, and lesser amounts of substituted thiophenes were identified in each tissue (3%). The bioaccumulation pattern exhibited after 30 days of exposure was clearly indicative of a classic threshold response. The absence of petrogenic hydrocarbons in tissue from the low and medium concentrations suggests that deputation mechanisms were capable of metabolizing aria/or eliminating parent hydrocarbons at these two exposure levels. Conversely, the high tissue

burden observed for fish in the high concentration indicates that extremely rapid hydrocarbon uptake exceeded the deputation capacity or threshold. This pattern of bioaccumulation suggests the deputation threshold resulting in bioaccumulation was somewhere between the medium (24.8 ppb) and high (177 ppb) concentration regimes.

Muscle and head/viscera bioaccumulation patterns became slightly divergent after 60 days of WSF exposure. No bioaccumulation had occurred in muscle tissue from northern anchovy exposed to the low concentration regime, although accumulation of petrogenic hydrocarbons was evident in muscle tissue from fish in the medium concentration. In contrast to muscle tissue, head/viscera tissues exhibited parent hydrocarbon accumulation at both exposure concentrations. The bioaccumulation patterns exhibited by both target tissues after 60 days of exposure further support the threshold phenomena observed after 30 days of exposure. The absence of petrogenic hydrocarbons in muscle tissue from fish exposed to the low concentration suggests that deputation mechanisms continued to operate efficiently through 60 days of exposure. However, the 60-day exposure threshold was apparently exceeded at an exposure concentration somewhere between the low (5.8 ppb) and medium (24.8 ppb) concentration regimes. Head/viscera tissue burden data also indicated that the deputation threshold was exceeded prior to day 60, resulting in hydrocarbon accumulation within one or more of the organs comprising this tissue group. In the three cases where demonstrable bioaccumulation occurred, alkyl-substituted benzenes, indenes, and naphthalenes were the predominant constituents.

Consistent bioaccumulation patterns and threshold phenomena were evident over the 60 to 90 day exposure interval. Analysis of muscle tissue from organisms sacrificed at day 90 revealed that significant bioaccumulation had occurred at the low concentration regime, and that the mean tissue burden had increased by 40% in the medium concentration. These data indicate the skeletal muscle deputation threshold was exceeded between 60 and 90 days in medium (24.8 ppb) and low (5.8 ppb) concentration regimes. The relative distribution of petrogenic hydrocarbon components in both target tissues was virtually identical to that observed in all of the tissues previously examined. At the end of the 90-day exposure period the mean head/viscera tissue burden had doubled for the fish in the low concentration, with no increase observed in the medium concentration.

Following termination of the experiment after 120 days of exposure, mean tissue burden levels had increased significantly in both tissue groups (Figures II-21 and 11-22). The petrogenic hydrocarbon tissue burden of skeletal muscle in the low concentration increased 79% between day 90 and day 120 whereas the tissue burden increase was only 34% in the medium concentration regime. Substantial tissue burden increases also occurred in the head/viscera tissue at both concentrations, with parent hydrocarbon bioaccumulation increasing by 125% in the low regime, and nearly doubling in the medium regime. The hydrocarbon composition in both target tissues exhibited the typical profile of alkyl-substituted benzene, indene, and naphthalene components. The dramatic tissue burden increase in both target tissues at both exposure concentrations provides further evidence of diminished deputation efficiency with continued exposure.

The overall bioaccumulation patterns observed for both adult northern anchovy target tissue types were indicative of the classic threshold response. Such patterns suggest that chronically exposed northern anchovy have a finite

deputation capacity or threshold, below which parental petrogenic hydrocarbons can be virtually eliminated through metabolically mediated activities. However, continued hydrocarbon uptake beyond this threshold level results in the bioaccumulation of these compounds and their incorporation into various tissue compartments. Bioaccumulation data from this study clearly indicate that this threshold is a function of the interaction of exposure concentration and exposure duration.

Cumulative survivorship of adult northern anchovy was extremely high (>97%) in the control, low (5.8 ppb) and medium (24.8 ppb) regimes after 120 days of exposure. In contrast, survivorship was greatly reduced in the high (177 ppb) concentration regime. For example, nearly half of all fish in the high concentration had died after 30 days of WSF exposure, while all fish died prior to 60 days of exposure.

The effect of exposure concentration was clearly evident in this experiment, with extremely high mortality occurring only in the high concentration regime. The threshold exposure concentration resulting in significant anchovy mortality evidently occurred at a petroleum hydrocarbon concentration somewhere between 24.8 (medium) and 177 ppb (high). From these results, it is also apparent that exposure concentrations less than 25 ppb were truly representative of a sublethal exposure regime.

As indicated previously, many short-term (acute) exposure experiments have been conducted that demonstrate petroleum hydrocarbons are highly toxic to adult and juvenile fishes at ppm concentrations. However, no previous long-term (chronic) exposure studies have been conducted with northern anchovy or any other species at the extremely low exposure concentrations (<0.2 ppm) used in this study. Our findings suggest that adult northern anchovy, and possibly many other species, are much more sensitive to petroleum hydrocarbon exposure than previously realized.

Growth of adult northern anchovy was assessed at periodic intervals during the 120-day experiment using estimates of mean standard length and mean total weight. The mean standard length of test fish changed very little in the low and medium concentration regimes during the 120-day experiment, with increases of 1% to 3% depending on the exposure regime. Although high concentration fish experienced very high mortality during the first 30 days of exposure, no significant change was noted in their standard length.

In contrast to standard length, the mean total weight of adult northern anchovy increased markedly during the 120 day experiment. Although there were no statistically significant differences in weight gain between fish in the different exposure regimes, the cumulative percentage weight gain for medium concentration fish was lower (28%) than for fish in the low concentration (41%) and control (38%) regimes. The slower weight gain of medium concentration fish was evident after all exposure periods (i.e. 30, 60, 90, and 120 days).

These results suggest that WSF exposure particularly at the medium concentration (24.8 ppb), may have adversely affected northern anchovy growth. Studies by Kern et al. (1976b) and McCain et al. (1978) have demonstrated reduced growth of striped bass and English sole following long-term exposure (4 to 16 weeks) to much higher (ppm levels) petroleum hydrocarbon concentrations. McCain et al. (1978) suggested that weight loss during hydrocarbon exposure was due to a higher level of stress. Conversely, Kern et al. (1976b)

attributed striped bass weight loss to both impaired food localization and an increased energy requirement for hydrocarbon metabolism and deputation that diverted energy from growth processes. Such a mechanism may explain reduced growth of adult anchovy in the medium concentration regime, since the onset of bioaccumulation occurred earlier and bioaccumulation reached higher levels in fish from the medium concentration. The diversion of assimilated energy from growth to metabolic activities relating to deputation can be expected to reduce both energy available for reproduction and stored energy reserves. Such metabolic changes may adversely affect reproductive success, reduce overall fitness, and possibly contribute to reduced survival under poor feeding conditions.

Northern anchovy tissues evaluated histologically for evidence of pathology included gill, heart, liver, pancreas, kidney, interrenal gland, spleen, stomach, pyloric caecum, intestine, gonad, skeletal muscle, skin, brain, and eye. The only change observed in any tissue due to NSF exposure was non-specific chemical damage to the gill tissue of fish in the high concentration. Gill damage was characterized by mild inflammation, thickening, focal fusion of the tips of secondary lamellae, and an increase in numbers of eosinophilic granular cells at the bases of the secondary lamellae. Such changes have frequently been described before relative to injury by petroleum products and other injurious chemicals (Ribelin and Migaki 1975, Roberts 1978, Cech 1983).

Although a few studies have examined the histological effects of oil exposure on adult fishes, their observations have been equivocal, perhaps due to varying experimental conditions. For example, Payne et al. (1978a) reported no evidence of pathology in several tissues (liver, kidney, heart, spleen, gonad, gill, muscle or gut) of cunner that were chronically exposed to Venezuelan crude for six months. In contrast, McCain et al. (1978) found strong evidence of liver pathology, including extensive hepatocellular lipid vacuolization, after only one month of exposure to oiled sediments (400 to 700 micrograms/g). No pathological effects, however, were found in the spleen, kidney, intestine, ovary, fin, gill or skin tissues. Observations of extensive liver damage in 'starry-flounder were also noted by Whipple et al. (1978) after only 5 to 7 days of exposure to Cook Inlet Crude WSF at concentrations intermediate (100 to 200 ppb) between the medium and high concentration regimes used in our experiments.

3. California Mussel

a. Embryo Experiment

Early life history stages of bivalves are generally regarded as more sensitive than adults to petroleum contamination (Neff et al. 1976a). Laboratory studies of embyro, larval and juvenile bivalves have provided oil exposure data supportive of the generalization (Renzoni 1975, Byrne and Calder 1977, Keck et al. 1978). The nature and intensity of oil exposure effects are dependent on numerous biological and exposure variables, including the organism studied, oil tested, dosing level(s), and duration. In the present study, exposure of Mytilus californianus embryos to three water soluble fraction (WSF) concentrations of Santa Barbara crude oil under static conditions produced significant new findings. These included sublethal effects on growth and development and larval mortality at real-world concentrations.

Lethal and sublethal responses of M. californianus embryos were dependent on test solution concentration. The order of magnitude differences in hydrocarbon exposure levels produced clearly distinguishable differential responses. Embryos exposed to the high concentration (360 ppb) generally exhibited the greatest intensity and number of effects, while the responses of embryos in the low (6.6 ppb) and medium (47,1 ppb) concentration regimes were less pronounced and varied. The present study did not address the question of deputation; however, numerous studies on adult organisms have shown that deputation processes do not remove all associated hydrocarbons (Nunes and Benville 1979, Neff et al. 1976b).

Survivorship of M. californianus larvae was assessed after 72 hrs of embryo exposure to WSF. Mortality was highest in the high concentration regime, while the number in low, medium and control exposure regimes we-e minimal and comparable (Table II-77). Although survival was assessed after 72 hrs of exposure, it is possible that mortality may have begun earlier. Byrne and Calder (1977), for example, noted that embryonic development in Mercenaria mercenaria had ceased within 3 hrs of exposure to 11.5 ppm No. 2 fuel 011. lthough mortality was significantly greater in the high concentration regime, the magnitude of mortality was relatively low (26%). Renzoni (1977) reported comparable mortality for Crassostrea virginica and Mulinia lateralis embryos exposed to oil concentrations as high as 1 ppt (1 ml/liter). Renzoni also reported significant sublethal effects due to oil exposure.

Although acute mortality is dramatic and of concern, sublethal effects that affect eventual development and survival are equally important. Sublethal effects of WSF exposure on M. californianus embryos were assessed through the evaluation of bioaccumulation, progress and completeness of development, and growth. In general, sublethal effects were more frequent and pronounced in embryos exposed to the highest concentration (360 ppb).

The extremely small size and limited tissue mass of the embryos required use of a radioactive tracer (^{14}C-naphthalene) to monitor hydrocarbon bioaccumulation. Estimation of bioaccumulation based on this technique was conservative since naphthalene was only a minor constituent of the complex WSF. We assume that the uptake proportions in adult tissues are similar to those in embryo tissues. Bioaccumulation of petroleum hydrocarbons was significantly higher in embryos exposed to the medium and high concentration regimes than in the control (2.8 ppb) or low concentration (6.6 ppb) regimes. Hydrocarbon bioaccumulation levels for animals in the medium and high exposure regimes represented a tenfold increase over control levels. Although other studies have not quantified uptake in molluscan embryos, the effects of high petroleum tissue burdens have been expressed in various ways including delayed or impeded development and growth.

Delayed or abnormal development increases the time that embryos remain in early development stages. No literature is available to indicate the relative vulnerability of a trochophore versus the later veliger stages; however, it is probable that earlier stages are more vulnerable to biological (i.e. predation) as well as environmental (i.e. physical/chemical) factors. In nature such vulnerability undoubtedly results in reduced survivorship for individuals as well as species. The number of embryos exhibiting depressed or delayed development was significantly elevated in the high concentration exposure regimes, compared to all others (Table II-78). This delay was phenotypically expressed in the high number of small embryos which had not developed a larval shell (i.e. Prodissoconch I Shell) after 72 hrs of

exposure. Since the experiments were terminated at 72 hrs, it was not possible to predict whether the embryos would have ever completed normal development. Renzoni (1975) found that some embryos of Mulina lateralis and Crassostrea virginica failed to complete normal development and further that the incidence of incomplete development increased with higher oil concentrations (i.e. 10 and 100 ppm and 1 ppt). The incidence of delayed embryo development in his experiments was significantly higher than that observed during this study. However, it should be noted that the "high" concentration exposure level from the present study (approximately 360 ppb) was less than one-half of Renzoni 's "low" concentration.

Growth characteristics of larvae developing from embryos exposed to petroleum have been monitored to assess sublethal effects. Slow growth and abnormalities of the shell and viscera were reported in a study of M. lateralis and C. virginica (Renzoni 1977). In the present study, larval M. californianus developing from embryos exposed to Santa Barbara crude WSF exhibited significantly less growth than control organisms. Veliger shell abnormalities were also recorded including irregular profiles and thickness. Shells of straight hinge veligers from exposed animals were significantly thinner than controls. In addition a large number of veligers in the high concentration regimes failed to develop prodissoconch shells. This condition appeared to be a true deformity and not just the result of delayed development. Support for this observation was derived primarily from the appearance of irregular rudiments of veliger shells or by shells so thin and reduced that they were barely detectable under the microscope. Growth effects such as these can be interpreted as environmental liabilities, since abnormal larvae are probably more susceptible to predation, bacterial infection, and other environmental stresses.

b. Larval Experiment

Embryos of Mytilus californianus which had developed in control seawater for approximately 72 hrs were transferred into experimental aquaria for the larval experiments. All veligers had reached the early straight hinge stage after 72 hrs and were of comparable size. Larval experiments lasted 24 days during which time various sublethal and lethal effects were monitored. Bioaccumulation of hydrocarbons and larval survival were correlated with exposure dose and duration. Sublethal effects evident in the higher concentrations included depressed growth, but abnormal veliger development was not evident.

Bioaccumulation of petroleum hydrocarbons, as in the embryo experiments, was monitored through uptake of ^{14}C-Naphthalene since limited tissue mass precluded direct measurement of hydrocarbon body burdens. Bioaccumulation was proportional to concentration and duration of hydrocarbon exposure. Initial hydrocarbon uptake by larvae in the medium (approximately 80.5 ppb) and high (approximately 559 ppb) exposure regimes was significantly higher than for animals in the low (approximately 14.2 ppb) and control regimes (Figure 11-25). Elevated hydrocarbon body burdens resulted in extremely high veliger mortality in the high concentration regime after 11 says of exposure. Through the remainder of the experiment, veligers in all regimes (including controls) exhibited continual hydrocarbon uptake with no evidence of an asymptote. This continual uptake ultimately caused significant veliger mortality in the medium concentration regime. Limited uptake by control animals suggests that some contamination of control aquaria occurred. One possible explanation is that

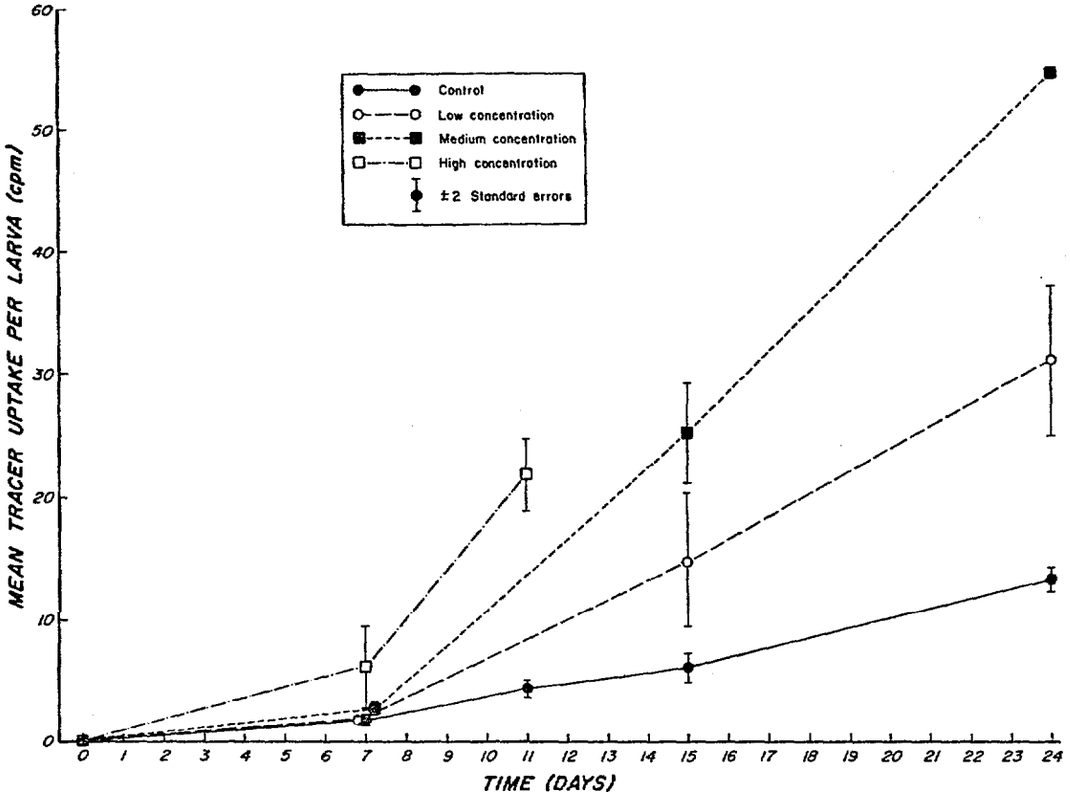


Figure II-35. Mean radioactive tracer uptake by California mussel veligers following 7, 11, 15, and 24 days of test solution exposure.

naphtha lene may have evaporated from experimental aquaria and redissolved into the control aquaria. Given the open nature of the flow-through system, this pathway is not unreasonable.

Continued hydrocarbon uptake after day 11 by experimental animals in the medium concentration regime did not result in complete mortality even though tissue hydrocarbon burdens exceeded those levels which caused death in the high concentration animals by day 11. This suggests that gradual bioaccumulation, although resulting in some mortality, permitted the test organisms to acclimate to the presence of petroleum hydrocarbons. Older larvae apparently were able to deal with the stress of oil contamination more effectively than younger veligers.

Marine invertebrates produce large numbers of larvae to ensure that some survive the vagaries of early development. Wells (1977) discussed how age class density can function as a refuge from predation. Other natural variables including physical and chemical factors also exist which impose additional survival pressures on developing individuals. In the present study moderate densities (approximately 21.7 larvae/ml) of M. californianus were exposed to the various hydrocarbon solutions. Veliger mortality was high in control and allWSF exposure regimes reflecting, in part, the high mortality which occurs naturally. Natural survivorship data are lacking for M. californianus;

however, Spight et al. (1974) and Spight (1975) reported 90 to 99% natural mortality in their study of gastropod larvae, and Mileikovsky (1971) reported survivorship values of 2% and 0.5 to 9% for Crassostrea gigas. Control larval mortality in the present study was 99% and approximates-values reported above. This suggests both that experimental survivorship was not unusually low and that the experiment approximated natural conditions. In spite of the high natural mortality, a clear inverse relationship was evident between larval survival and test solution concentration (Figure 11-36). Veligers exposed to the high concentration (approximately 559 ppb) regime survived for only 11 days. Larvae in the control (approximately 4.4 ppb), low (approximately 14.2 ppb) and medium (approximately 80.5 ppb) concentration regimes survived until the experiments were terminated at day 24, but with significant reduction in numbers. By day 24 mortalities in the medium exposure regime were significantly higher than in the low or control aquaria where they were roughly comparable. Since the high concentration was lethal in a relatively short time period, and significant mortalities occurred in medium levels when exposure periods were increased, the results clearly demonstrate the relationship and effects of exposure concentration and duration.

Developmental characteristics such as size and morphology have been used to monitor the sublethal effects of hydrocarbon exposure (Renzoni 1977, Byrne and Calder 1977). Growth of M. californianus veliger shell length and height were measured at days 7, 11, 15 and 24. Growth in terms of shell length and height was affected in a similar manner by hydrocarbon exposure (i.e. if growth in shell length was depressed by hydrocarbon exposure then shell height was also depressed). As a result the separation of shell length and height in

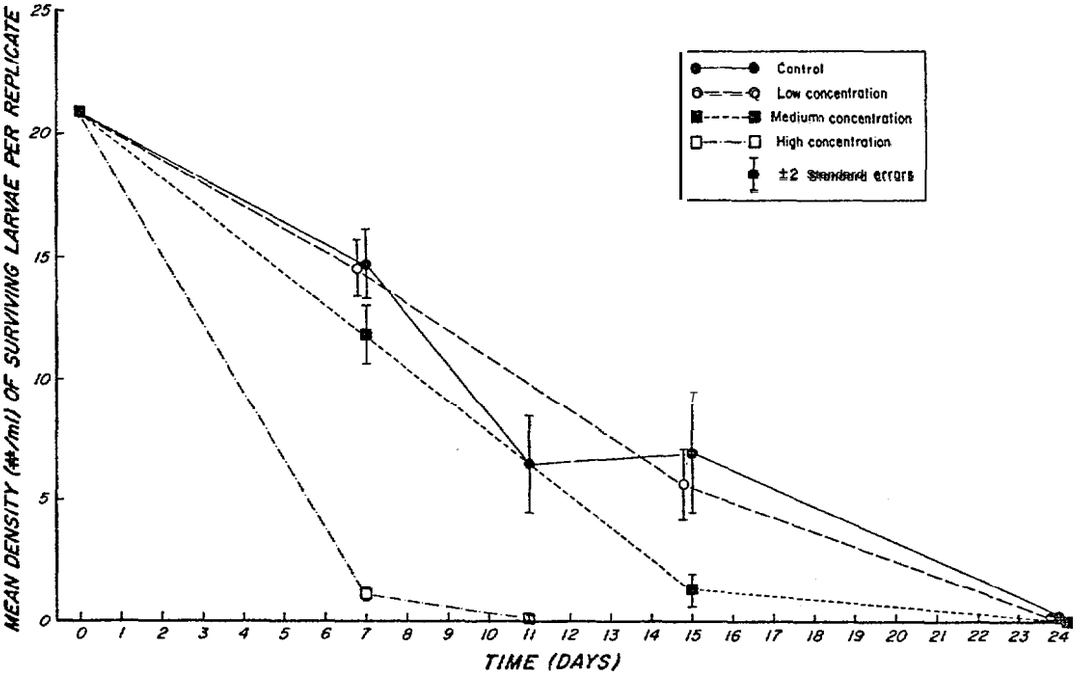


Figure II-36. **Mean density of surviving California mussel veligers following 7, 11, 15, and 24 days of test solution exposure.**

the discussion of growth was replaced by the single term "size". Veliger size data revealed some interesting results which preclude simple generalities. Significant size differences were noted between control and WSF-exposed larvae; however, these differences were both positive and negative during the early larval measurement period (i.e. days 7 and 15) (Figures II-23 and II-24). At day 7 growth in the high concentration larvae was significantly depressed when compared to control animals on day 7 and at day 11 when the high concentration experiments were terminated because of extremely high mortality. By contrast, the control veligers were significantly smaller than low and medium exposure regime larvae at day 7, and control concentration larvae remained smaller than medium concentration veliger on day 15. These observations suggest that enhancement of growth occurred in the lower hydrocarbon exposure regimes.

The M. californianus veligers were maintained in flow-through regimes with unfiltered seawater, since their food requirements were met by bacteria, algae and detrital material in the seawater. Many bacterial, algal, and fungal strains are known to thrive on organic material contained in crude oil (Cooney and Walker 1972, Soli 1972, Mechalas et al. 1972). Adult mussels feed on bacteria, detrital, and algal material (Fox 1936, Fox and Coe 1943) and similar food habits, although smaller in size (<10 micron), are described for bivalve larvae (Bartlett 1972, Walne 1974). It is possible that bacterial, algal, and fungal populations in the system seawater were enhanced by oil in the exposure aquaria and, thus provided additional food to larvae held in those regimes. Examination of veliger size data after 24 days of exposure, however, showed that control larvae were significantly larger than veligers from either the low or medium concentration regimes. This probably related to the influence of bioaccumulated hydrocarbons which increased significantly through time and undoubtedly affected growth even though "additional" food may have been available. Byrne and Calder (1977) also reported depressed growth in quahog (Mercenaria sp) larvae; but failed to report enhanced larval growth. Enhancement of growth may not have been detected since growth was measured only at the end of the experiment. The depressed growth as discussed by Byrne and Calder "casts doubt on the ability of larvae to continue to survive even if the oil stress were removed or if they survived on their capability for successful reproduction".

Whole mounted veligers were examined for microscopic evidence of delayed or abnormal development at days 7, 15 and 24. In contrast to the embryo experiment findings, no abnormal development was detected. As discussed above, size differences were observed between larvae from the various exposure regimes. The absence of veliger shell abnormalities suggests that the most critical period for veliger protoconch shell development was within the first 72 hrs. This conclusion is based on the previously discussed results of the embryo experiment which included shell abnormalities after 72 hrs of exposure. Since the "larval" experiments were performed on approximately 72 hr old larvae without previous exposure history and no malformations were detected after 24 days, the critical period apparently is within the early embryo/early veliger period. These findings suggest that the early embryo] early veliger (i.e. <72 hrs old) stages are more sensitive to hydrocarbon exposure and are important for normal protoconch shell development.

c. Adult Experiment

Numerous studies have examined the effects of oil exposure on adult bivalves including clams, oysters and mussels (Riley et al. 1981, Sabourin and

Tullis 1981, Clark and Finley 1975, Neff et al. 1976). Studies conducted by Nunes and Benville (1978) on the Manila clam Tapes semidecussata and Kanter (1971 and 1973) on the California mussel Mytilus californianus have demonstrated acute toxic effects of crude oils similar to those tested in this study. Other studies performed on the same organisms, with different oils and exposure regimes, have reported a variety of sublethal effects. Despite the number of studies performed, differences in oil type, dosing, and exposure regime between studies preclude generalizations regarding oil effects on bivalve mollusks.

Long-term static oil toxicity studies conducted by Kanter (1971 and 1973) on Mytilus californianus found adults of this species to be extremely susceptible to toxic effects at higher concentrations (i.e. 1 x 10^5 ppm) and considerably more resistant at lower doses (e.g. 1 x 103 ppm). In the present study mortalities were minimal. Even in the highest concentration (e.g. 413 ppb regime) no statistical ly significant differences were detected between control and experimental animal survivorship. Although no lethal effects of the Santa Barbara crude oil were detected, significant bioaccumulation of petroleum hydrocarbons did occur and sublethal effects were observed.

Uptake and deputation of petroleum hydrocarbons in mollusks have received considerable study (Nunes and Benville 1979, Stainken 1978, Stegeman and Teal 1973). These studies conclude that deputation occurs rapidly with higher molecular weight aromatic hydrocarbons being released more slowly than lower weight species (Neff et al. 1976a). However, the purging process was not 100% efficient and significant quantities of contaminating hydrocarbons remained in animal tissues (Blumer et al. 1970, Vandermeulen et al. 1978, Fong 1976, Boehm and Quinn 1976). Fong (1976) found a direct correlation between tissue lipid content and hydrocarbon bioaccumulation. In addition, the presence (or absence) of an enzyme system capable of breaking down or metabolizing hydrocarbons in part determines the quantities and effects of accumulated hydrocarbons (Vandermeulen and Penrose 1978, Roesijadi and Anderson 1979, Bayne et al. 1979, Moore et al. 1978). Persistence of petroleum residues in tissues is particularly important since incomplete elimination allows hydrocarbons to be passed on to consumers, including man.

Past bioaccumulation and deputation studies generally analyzed entire organisms (i.e. no separation of various organs or tissues). With few exceptions these studies examined effects of single compound exposures and analyzed the total tissue mass for these compounds (e.g. benzo-a-pyrene, mineral oil, toluene, naphthalene) (Fossato and Canzonier 1976, Dunn and Stitch 1976, Riley et al. 1981). In the present study evidence of a complex hydrocarbon mix (i.e. WSF of Santa Barbara Crude) was monitored i n target tissues including the gill, gonad, muscle, and digestive gland. However, no deputation studies were conducted. Experiments with Mytilus edulis, (Dunn and Stitch 1976, Lee et al. 1972, Fossato and Canzonier 1976) have shown that significant quantities of hydrocarbons were retained long after deputation ceased. Further, Dunn and Stitch (1976) report that petroleum hydrocarbons bioaccumulated during long-term chronic exposure were released much more slowly and were ultimately retained in higher levels in the tissues. This information suggests a similar response is likely for M. californianus and that the tissue burden patterns detected are likely to persist, although absolute quantities may decrease if deputation and/or metabolization occur.

Petroleum derived hydrocarbons were not detected in any of the five target tissues from adult California mussels collected for establishment of

background tissue burdens. These data indicate that organisms collected for use in the toxicity study were not contaminated with petrogenic hydrocarbons at the initiation of the bioassays. Additionally, analysis of target tissues from control mussels sacrificed at the termination of the bioassay revealed no detectable levels of petrogenic hydrocarbons. The absence of petrogenic hydrocarbons implies that control organisms were not contaminated over the course of the toxicity study.

No parental petrogenic hydrocarbons were detected in retractor/adductor muscle tissue from any adult mussel exposed to the three WSF regimes of Santa Barbara crude. In contrast, Fong (1976) found Kuwait crude residues (483 ug/g wet weight) in the foot and adductor muscle of the clam Mya arenaria, after 10 days of exposure. Data from this study suggest that muscle tissue either did not take up hydrocarbons to an appreciable degree; or that if uptake occurred, parental hydrocarbons were readily metabolized and eliminated. Similarly, histological examination of muscle tissue from WSF-exposed organisms revealed no significant pathology. The histological and bioaccumulation data suggest that exposure to water soluble crude oil constituents does not result in a readily observable impact on adult M. californianus muscle integrity.

Relatively low hydrocarbon tissue burden levels were observed in gill tissue from adult mussel exposed to both the medium and high level dosing solutions. At both exposure levels two isomers of dimethylphenanthrene were the only petrogenic hydrocarbons detected. This observation is in sharp contrast to those of Fong (1976) who reported much higher bioaccumulation in clam gill tissue. A possible explanation for part of this difference may lie in the relative lipid content of the tissues, and probably reflects the higher lipid content of clams compared to mussels. It is possible that petrogenic hydrocarbons transferred across gill membranes were transported to other mussel tissues where accumulation and/or metabolic conversion occurred. These suppositions are strengthened by the fact that only alkyl phenanthrenes were identified in mussel gill, whereas the more typical alkyl benzenes and naphthalenes were absent,

Histological examination of gill tissue from WSF-exposed organisms revealed no significant pathology, suggesting that chronic exposure to petrogenic hydrocarbons did not result in significant tissue damage. However, other researchers have indicated that physiological functioning of the gills in respiration and feeding is indeed affected by bioaccumulated hydrocarbons. Stainken (1978) reported accelerated respiration in clams (Mya arenaria) exposed for 28 days to 10 ppm No. 2 fuel oil. Further he found that higher concentrations (e.g. 10,100 ppm) resulted in significantly depressed oxygen consumption and gill function. Sabourin and Tunis (1981) found that aromatic hydrocarbons including benzene and toluene in concentrations ranging from 10 to 100 ppm significantly reduced oxygen consumption in M. californianus. In addition to organ-specific physiological responses which must be considered, the consequences in terms of energy costs to the organism as a whole involved in blocking hydrocarbon transfer across gill membranes and/or deputation activities remain unclear.

Petrogenic hydrocarbons accumulated in adult mussel ovary tissue at all three exposure levels, although only a trace amount was detected in the low concentration regime. Bioaccumulation was significant; higher at both the medium and high exposure levels. In the medium concentration, substituted benzenes and thiophenes were the predominant species identified; whereas

alkyl naphthalenes and benzenes contributed most substantially at the high exposure level. One compound, dimethylnaptthol (2,3-B)-thiophene, accounted for over 50% of the total tissue burden at the medium exposure level.

The high bioaccumulation levels observed in ovary tissues from mussels in the medium and high concentrations are probably related to the high lipid content of those tissues. Analysis of ovary tissue revealed a gross lipid content of approximately 0.2%, a value somewhat less than that reported by Fong (1976) for Mya arenaria gonads. Lipid material within ovarian tissue may thus serve as a depot where petrogenic hydrocarbons are sequestered and the amount of material accumulated probably will be associated with the reproductive state of the individuals. This partitioning process has been observed to occur in ovarian tissue of marine polychaetes exposed to the WSF of No. 2 fuel oil (Rossi and Anderson 1977). These investigators suggest that during oocyte maturation within the female, oocytes constitute a stable storage compartment, since lipids in oocytes are metabolically unavailable to the female. Histological examination of adult mussel ovary tissues from this study tends to support this interpretation, since no significant pathology was observed in gonadal tissue from the WSF-exposed mussels.

These data suggest that accumulated hydrocarbons were effectively sequestered in the lipid depot and did not result in apparent damage to ovarian tissue or developing oocytes. Although no apparent impact was observed on adult females, bioaccumulation of petrogenic hydrocarbons in ovarian tissues could have a significant impact on the offspring of contaminated females. Similar studies conducted with polychaete worms indicate that hydrocarbons accumulated by gravid females were passed to offspring through storage in yolk material (Rossi and Anderson 1977). In addition, the embryo experiments of the present study suggest that the early 72 hrs of development in previously non-exposed animals were critical with regard to veliger development. It thus seems likely that embryonic utilization of contaminated yolk material could result in adverse impacts on embryonic development, hatching success and/or larval development in the successive generations.

Bioaccumulation of petroleum hydrocarbons in the digestive gland has not received much attention although Fong (1976) found moderate (399 µg/g wet weight) quantities of aromatic hydrocarbons derived from Kuwait crude in the clam Mya arenaria. Extensive bioaccumulation of hydrocarbons was recorded in digestive tissue from adult mussels exposed to the medium and high level dosing solutions. Petrogenic hydrocarbons were not detected at the low exposure level. The bioaccumulation pattern observed in this tissue was somewhat unique, in that alkyl-substituted benzenes were not detected, but substantial levels of substituted thiophenes and phenanthrenes were identified. At the medium exposure level, substituted thiophenes were the predominant species accumulated; a pattern similar to that observed in mussel ovary tissue. At the high exposure level the tissue burden was roughly evenly divided among alkyl-substituted naphthalenes, thiophenes and phenanthrenes.

The exact mechanisms of hydrocarbon accumulation in the digestive tissue of marine bivalve mollusks are largely undocumented. However, Stainken (1976) used dyed oil and showed that oil globules were handled like food particles by clams and ingested. In addition, direct membrane absorption probably occurred. Unfortunately, tissue mass limitations precluded gross lipid analysis, confounding speculation regarding the fate of accumulated hydrocarbons. It seems likely however, that lipid depot serves as one

potential accumulation compartment, since Fong (1976) reported that the lipid content of this organ in clams was approximately 2.5 times greater than in gill or gonad tissue.

Histological examination of digestive tissue from experimental organisms revealed pathological changes in WSF-exposed organisms. The most pronounced pathological effect was infiltration of inflammatory cells in spongy connective tissue. The infiltrates consisted of acidophilic granular amoebocytes and macrophages indicative of cellular damage and stress. The presence of such cells, in conjunction with the high tissue burden levels, suggests that chronic exposure to the medium and high level test solutions may have resulted in impaired digestive tract function and significant stress to oil-exposed mussels.

Testes from exposed adult California mussels exhibited the greatest accumulation of petrogenic hydrocarbons at the high dosing level, and most typical overall accumulation pattern. Figure II-37 presents a graphical summary of the type and level of petrogenic hydrocarbons identified in testis tissue from experimental organisms. As this figure indicates, only trace levels of petrogenic hydrocarbons were detected at the low exposure level. At the medium dosing level substantial bioaccumulation occurred, with alkyl-substituted benzenes and naphthalenes the predominant hydrocarbons identified. Bioaccumulation was extensive at the high exposure level, including significant contributions from all four hydrocarbon categories. Alkyl-substituted phenanthrenes accounted for over 16% of the total tissue burden, and mean concentrations of individual phenanthrenes were as high as 550 rig/g. Estimated bioaccumulation factors were observed to be as high as 10600 for substituted thiophenes, 2780 for alkyl benzenes, 2960 for naphthalene derivatives, and over 4400 for alkyl phenanthrenes.

A possible explanation for the relatively extensive bioaccumulation observed in testes rrom WSF-exposed animals (particularly at the high exposure level) is the high lipid content. As previously stated, a high correlation exists between tissue lipid content and quantity of hydrocarbons bioaccumulated (Stegeman and Teal 1973, Fong 1976). Gross lipid analysis of tissue from organisms sacrificed at the termination of the study revealed adult mussel testis contained roughly 1.2% lipids, a value six times greater than mussel ovary tissue. Although these data seem intuitively anomalous, the analytical data suggest that gonadal tissue components associated with sperm production were particularly lipid rich. Lipid content is also likely directly related to the reproductive stage of the organism.

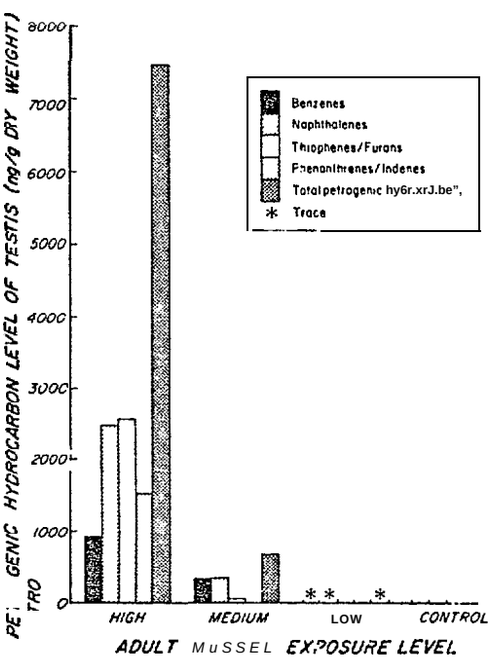


Figure II-37. Petrogenic hydrocarbon tissue burden profile of adult California mussel testis.

It is thus possible that the hydrocarbon tissue burdens observed reflected differences in lipid content as a function of differences in reproductive cycles between male and female organisms over the course of the bioassay or near its termination.

Regardless of the reason for the high lipid content, it seems evident that petrogenic hydrocarbons were sequestered in testis lipid depot. However, the nature and extent of the impact of petrogenic hydrocarbon accumulation in mussel testis tissue are unclear. Histological examination of tissue from WSF-exposed organisms revealed no detectable pathology at any exposure level. All tissues examined appeared normal in all regards and contained mature gametes. There was thus no direct evidence of an adverse impact on adult organisms, but tissue burdens of the magnitude observed could potentially result in impaired sperm mobility, decreased longevity and/or reduced fertilization success (Renzoni 1975).

A graphical summary of the total petrogenic hydrocarbon tissue burden for each of the adult M. californianus target tissues is presented in Figure II-38. Examination of the data contained on this graph reveals several distinct trends. The bioaccumulation pattern depicted is again indicative of a threshold effect (discussed for the California halibut and northern anchovy previously). Exposure to the low level test media resulted in very little accumulation of parent petrogenic hydrocarbons. However, with the exception of the retractor/adductor muscle, all target tissues showed significant bioaccumulation at the medium and high exposure levels. In three of the target tissues (gill, digestive and ovary) the hydrocarbon tissue burden was essentially equivalent at the medium and high dosing levels. These data suggest that deputation mechanisms were effective in metabolizing and/or eliminating parent hydrocarbons at the low exposure level. However, it appears that the rate of hydrocarbon uptake surpassed the organisms' ability to depurate at the medium and high dosing levels. These results contrast somewhat with those of Vandermeulen and Penrose (1978), who reported the absence of aryl-hydrocarbon hydroxylase (AHH) and N-demethylase enzyme activity in bivalves including Mytilus californianus. They suggest that this inability to metabolize accumulated hydrocarbons permits transfer of unaltered hydrocarbons to the food chain. The results of the present study are not totally incompatible with their findings since in the higher exposure regimes hydrocarbons did accumulate. Vandermeulen and Penrose's concentrations (e.g. 4.3 to 24.9 ppm) exceeded even the highest concentration of this study and suggest that they may well have exceeded their test organisms'

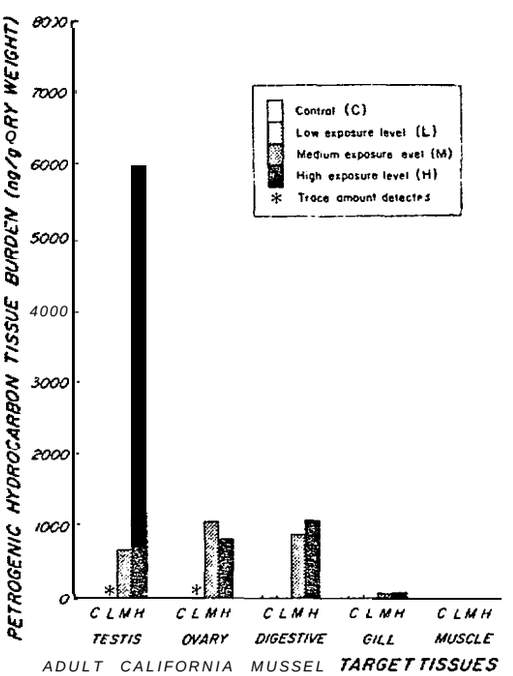


Figure II-38. Total petrogenic hydrocarbon tissue burden of adult California mussel.

ability to metabolize lower concentrations of hydrocarbons. However, one would have expected some evidence of AHH even if it was not effective in metabolizing all petroleum hydrocarbons. Studies conducted on other mollusks including gastropods (Moore et al. 1978) and mollusks (Bayne et al. 1979) found evidence of enzyme detoxification systems. The bioaccumulation pattern described for the present study indicates that a deputation threshold was reached for four of the five target tissues at a level between the low (13 ppb) and medium {48 ppb) exposure regimes. The nature of this enzyme system requires further study.

In general, bioaccumulation was more extensive at the medium exposure level in target tissues from M. californianus than in either of the two vertebrate species investigated. The maximum level of accumulation in adult mussel target tissues from the medium dosing level was approximately twice that observed for similarly exposed adult anchovy, and roughly seven times that observed for adult halibut. These data are consistent with those from other studies, which have indicated that marine mollusks generally accumulate hydrocarbons to higher levels than other marine organisms (Hansen et al. 1978, Lee et al. 1978, Boehm and Quinn 1977). The enhanced accumulation may be due to the inability of mollusks to metabolize hydrocarbons to more water-soluble and readily excreted polar metabolizes (Lee et al. 1972). However, the similarity in mussel digestive, ovary and gill bioaccumulation levels is somewhat perplexing. Based on these three tissues, it would seem that uptake rate is independent of the aqueous exposure level once the threshold is reached. Contrarily, the testis displayed a more traditional bioaccumulation pattern in which accumulation at the high exposure level was approximately 10 times greater than at the medium exposure level. A possible explanation is that the high lipid content of the testis served as a particularly effective partitioning medium for sequestering parent hydrocarbons, resulting in a more direct correlation between uptake and aqueous exposure level.

One additional trend was apparent in the adult mussel bioaccumulation data. In both vertebrate studies, alkyl-substituted phenanthrenes were identified in only one of the eight target tissues examined, and in this tissue (adult halibut gill) only a trace amount was detected. In the adult mussel study, alkyl-substituted phenanthrenes were identified in four of the five target tissues. The only tissue in which phenanthrenes were not identified was the retractor/adductor muscle, where no petrogenic hydrocarbons were detected. Alkyl-phenanthrenes were not only detected with high frequency in adult mussel tissue, but were also found at high concentration levels (individual specimens as high as 550 rig/g). This observation is consistent with previous studies utilizing various aromatic hydrocarbons and marine bivalve mollusks. In a study conducted by Neff et al. (1976) clams exposed to single-component solutions of four aromatic hydrocarbons (naphthalene, phenanthrene, chrysene and benzo(a)pyrene) were found to accumulate phenanthrene most readily. Similar data obtained by other investigators (Lee et al. 1972, Anderson 1978, Vandermeulen and Penrose 1978) suggest that marine bivalve mollusks have little ability to metabolize higher molecular weight, aromatic hydrocarbons.

In addition to organ-specific bioaccumulation and histological examination, sublethal effects on mussel growth and byssus thread production were monitored. Adult mussels were randomly assigned to experimental and control aquaria. Each animal was individually marked with a numbered reference tag and its shell height, length, and girth were recorded. Upon an animal's demise or

when the experiments were terminated, shell size parameters were again recorded. From these data growth in shell features was calculated. Mussels which survived showed growth in all three shell features during the 120 days. Shell length and height increased approximately 0.5 mm and girth approximately 0.7 mm. These growth rates are comparable to those reported in the literature (Bayne 1976). In contrast to the results reported for the mussel larval experiments, no significant differences in growth were detected between the control or any experimental animals. Few adult mussels died during the experimental period. Examination of these limited data suggested that exposure to WSF concentrations over the range of those tested did not adversely affect mussel growth.

Byssus thread production is vital to the survival of M. californianus since its primary habitat is the surf-swept rocky headlands along the exposed Pacific coastline. The byssus threads form a strong attachment between the mussel and substrate enabling the mussel to withstand the pounding surf, yet maintain a position in the water column with well-aerated water and a relatively continuous food source (at least during high tide). In nature, byssus threads are produced intermittently and usually in response to water movement and need for replacement of broken threads (Bayne 1976). This process requires the expenditure of both energy and raw materials by the organism. The effect of petroleum hydrocarbon exposure on byssus thread production has been examined in Mytilus edulis (Carr and Reish 1978, Martella 1973). Carr and Reish found the WSF of No. 2 fuel oil and Louisiana crude oil at concentrations of approximately 2 and 4 ppm produced enhanced byssus thread production during a 96-hr study, while at higher concentrations (approximately 8.7 and 19.8 ppm) for longer durations (approximately 14 days) byssus thread production was significantly depressed. In the present study, exposure concentrations were an order of magnitude below those of Carr and Reish and the exposure duration lasted up to 120 days. Weekly checks showed increased byssus thread production during the first three weeks in all experimental and control aquaria; however, animals in the high concentration (and occasionally in the medium concentration) produced significantly fewer threads than animals in any other regime. This pattern persisted throughout the remainder of the experiment, although byssus thread production in all animals dropped off from the initial high levels. The overfill decrease in byssus thread production was followed by a leveling-off of production, a pattern which continued until the experiments were terminated. The depression of byssus thread production in the high (approximately 413 ppb) and occasionally in the medium (approximately 48 ppb) concentration regimes suggests that this energy requiring activity was substantially reduced in the presence of petroleum hydrocarbons. This probably indicates a diversion or conservation of energy to deal with the sublethal effects of oil exposure. Further, the ecological implications of this reduction include increased vulnerability to surf and surge action as well as predation. Also of interest with regard to byssus thread production was the reduced level of production during the later weeks of the experimental period. Since the animals were basically maintained in tanks devoid of surf and surge activity, the lower level of byssus thread production may in part reflect this less physically stressful setting of the test aquaria. However, the fact that byssus threads were cut and counted weekly and the animals were handled should have provided enough physical stress to initiate thread production.

The adult mussel studies have provided substantial evidence of sublethal hydrocarbon effects. The term sublethal is perhaps improper, since ultimately these responses to hydrocarbons can overburden the organism's physiological

systems and substantially reduce survival potential through the organism's lifetime. Certainly impaired gill function can inhibit respiration and/or feeding. Gonad hydrocarbon burdens may, as Renzoni (1977) demonstrated, reduce gamete viability and thus reduce reproductive success of the species. Finally, increased vulnerability to natural stress (i.e. such as being ripped off traditional substrates when byssus threads do not hold) represents a threat to survival of individuals and populations.

E. CONCLUSIONS

The assessment of potential impacts from oil and gas operations in the marine environment is one of the principal tasks facing decision makers in the Minerals Management Service and the Department of the Interior. As a result. of recent discoveries, it is projected that one billion barrels of oil will be produced in the Santa Maria Basin and Santa Barbara Channel in the next two decades. To better assess the potential environmental impacts of this development, decision-makers require information on the potential impacts of an oil spill on the marine environment. The purpose of this study was to measure chronic impacts on representative fisheries resources from petroleum hydrocarbon levels typically occurring near oil spills.

Although petroleum hydrocarbon concentrations in the parts per million range have been measured near or immediately after a major spill event, the more commonly recorded concentrations near spills or in areas of chronic contamination are in the low-to-mid parts per billion range (Table I-l). Ambient water column concentrations rarely exceed the low parts per billion/ high parts per trillion range. Although one must consider the limitations of these measurements due to differences in analytical techniques, it is still reasonable to assume that petroleum hydrocarbon levels in the low-to-mid parts per billion range (such as those used in this study), are realistic for the marine environment after a major spill event.

Previous oil toxicity studies have demonstrated that exposure of marine organisms to oil or oil components at concentrations in the parts per million range resulted in direct mortality as well as serious sublethal effects. Results from our study, however, indicate that marine organisms in general, and early life history stages in particular, are much more sensitive to the toxic effects of oil than previously reported. In addition, this study indicated that apparent sublethal effects may ultimately result in death of organisms since they reduce the individual's ability to develop and feed normally.

The results from this laboratory study clearly demonstrated that early and adult life history stages of fish and shellfish experienced both lethal and sublethal effects following exposure to parts per billion of petroleum hydrocarbons. It is likely that individuals of the species tested, as well as ecologically-related species, will show similar responses if exposed to petroleum hydrocarbons under comparable conditions in the marine environment. Even though the species tested in this study occur in the region expected to experience future oil and gas development (i.e Santa Maria Basin/Santa Barbara Channel), the populations of these species occur over a much broader geographic area. For fish and shellfish species which are abundant and broadly distributed, the impacts of petroleum hydrocarbon exposure on local populations are not likely to be separable from other factors (e.g. predation, natural mortality, fishing pressure) that also influence these populations.

In contrast, fish and shellfish species that have more restricted geographic ranges or occur at lower population levels are likely at greater risk and may experience significant population impacts due to petroleum hydrocarbon exposure.

F. SUMMARY

SUMMARY OF FINDINGS

Test Oil Analyses

1) Santa Barbara crude oil is a complex mixed-base crude characterized by a diverse array of aliphatic and aromatic hydrocarbons, and a high organosulfur content ("sour" crude).

2) The composition of the water soluble fraction generated from Santa Barbara crude oil was dominated by lower molecular weight (5-12 carbon atoms) aliphatic, aromatic, and polar/heterocyclic hydrocarbons.

3) The composition of the water soluble fraction closely followed that predicted by aqueous volubility considerations; with polar and aromatic hydrocarbons most prevalent and at highest concentration levels.

4) Aromatic and heterocyclic hydrocarbons were identified most frequently in target tissues from exposed organisms. These compounds could be readily traced from the parental Santa Barbara crude, through the aqueous exposure media (kISF), to accumulation in target tissues.

California Halibut

Embryo Experiment

1) Halibut embryos exhibited marked bioaccumulation of petroleum hydrocarbons based on measurements of radioactive tracer uptake. Significant bioaccumulation was found in the medium (91.3 ppb) and high (761 ppb) concentrations.

2) Survivorship and hatching success of halibut embryos decreased with exposure to increasing WSF concentrations and tissue bioaccumulation levels. Survivorship and hatching success were significantly reduced in the medium and high concentrations.

3) Newly-hatched larvae from halibut embryos exposed to the nigh concentration were significantly reduced in size and characterized by a high incidence of bent notochords. The incidence of bent notochords was also significantly elevated for newly-hatched larvae from the low (10.2 ppb) and medium concentrations.

Larval Experiment

1) Halibut larvae exhibited significant petroleum hydrocarbon bioaccumulation in the low and medium concentration: based on measurements of

radioactive tracer uptake. Bioaccumulation was directly proportional to both exposure concentration and the duration of exposure.

2) Survivorship of larval halibut was markedly reduced in all WSF concentrations (10.5-606 ppb). Survivorship was inversely proportional to exposure concentration, bioaccumulation and the duration of exposure.

3) Growth rates of larval halibut were significantly reduced in all WSF concentrations (10.5-606 ppb). The onset of reduced growth was dependent on both exposure concentration and duration of exposure.

4) Halibut larvae in the medium and high concentrations were characterized by delayed and/or abnormal development. Such developmental anomalies of larvae in the medium concentration caused impaired feeding and eventual mortality.

Adult Experiment

1) All adult halibut target tissues exhibited marked bioaccumulation of petroleum hydrocarbon following exposure to the high (417 ppb) concentration. Bioaccumulation levels were highest in gill, liver and digestive tract tissues.

2) Survivorship of adult halibut. was reduced in all WSF concentrations (9.5-417 ppb), but was more pronounced in the high concentration. Survivorship was inversely proportional to both exposure concentration and duration of exposure.

3) Growth and/or condition of adult halibut was not adversely effected by WSF exposure.

4) Tissue pathology attributable to WSF exposure was only found in the gill and liver tissue of halibut exposed to the high concentration.

Northern Anchovy

Embryo Experiment

1) Anchovy embryos exhibited marked bioaccumulation of petroleum hydrocarbons based on measurement of radioactive tracer uptake. Bioaccumulation was significantly elevated in the medium (188 ppb) and high (367 ppb) concentrations.

2) Survivorship and hatching success of anchovy embryos decreased with exposure to increasing WSF concentrations and tissue bioaccumulation levels.

Larval Experiment

1) Anchovy larvae exhibited marked petroleum hydrocarbon bioaccumulation in the medium (85 ppb) and high (384 ppb) concentrations based on measurements of radioactive tracer uptake.

2) Survivorship of anchovy larvae was markedly reduced i n WSF concentrations ranging from 29.9 (low) to 384 (high) ppb. Survivorship was inversely proportional to exposure concentration and the bioaccumulation level.

3) Growth rates of larval anchovy were significantly reduced in the medium and low (29.9 ppb) concentrations, although the onset of reduced growth was dependent on exposure duration:

4) Anchovy larvae in the medium concentration were characterized by structural abnormalities including poorly developed jaws, reduced ossification and poorly developed pectoral fins. These abnormalities resulted in a failure to feed normally, and eventually caused mortality.

Adult Experiment

1) Both adult anchovy target tissues (i.e. muscle and head/viscera) exhibited marked bioaccumulation of petroleum hydrocarbons. Bioaccumulation levels were directly proportional to exposure concentration and duration of exposure.

2) Adult anchovy survivorship was significantly reduced only in the high (177 ppb) concentration. Mortality was negligible in all other concentrations.

3) Growth (in weight) of adult anchovy was markedly lower in the medium (24.8 ppb) concentration.

4) Gill tissue of adult anchovy in the high concentration was the only tissue found to exhibit pathology attributable to WSF exposure.

California Mussel

Embryo Experiment

1) Mussel embryos exposed to the high (360 ppb) and medium (47.1 ppb) test concentrations bioaccumulated significant quantities of petroleum hydrocarbon based on radioactive uptake.

2) Survivorship of mussel embryos after 72 hrs of exposure was significantly reduced in animals exposed to the high concentration of petroleum hydrocarbon test solution.

3) Veligers developing from embryos exposed to the high concentration petroleum hydrocarbon test solution showed an abnormally high incidence of developmental abnormalities, including deformed or missing shells.

4) Shells of all veligers developing from embryos exposed to all WSF concentrations (6.6 - 300" ppb) were extremely thin arid abnormally small.

Larval Experiments

1) Significant quantities of petroleum hydrocarbons measured by tracer uptake were bioaccumulated throughout the entire experimental period in all veligers tested.

2) Veligers exposed to the high (559 ppb) concentration lived for only 11 days. Larvae in the control (4.4 ppb), low (14.2 ppb) and medium (80.5 ppb) concentrations survived until the experiments were terminated at day 24, with animals in the medium concentration exhibiting a significant reduction in numbers.

3) Veligers exposed to all WSF concentrations (14.2 - 559 ppb) showed significant growth differences throughout the experimental period. Those in the high concentration were significantly smaller by day 11. Veligers exposed to medium and low concentrations were abnormally small when measured after 24 days of exposure.

4) Shell thickness for veligers developing in petroleum test solutions appeared normal after 24 days of exposure to petroleum hydrocarbons.

5) The early embryo/early veliger (i.e. <72 hrs) mussel life history stages are extremely critical to normal protoconch shell development and sensitive to petroleum hydrocarbon exposure.

Adult Experiment

1) No hydrocarbons of petroleum origin were found in muscle (retractor and adductor) tissues of adult mussels following 120 days of exposure to test solutions (12.7 - 413 ppb). However, significant quantities of petroleum hydrocarbon were found bioaccumulated in gill tissue, digestive gland, ovary, and testes of exposed organisms.

2) Adult mussel survivorship in control animals and those exposed to petroleum hydrocarbons was high throughout the entire experimental period.

3) No statistically significant differences in growth (i.e. increases in shell length, height, or width) between control or experimental groups were detected during the entire experimental period.

4) Periodic evaluation of byssus thread production in adult mussels revealed that animals in the high concentration [(413 ppb) (and occasionally in the medium (48.1 ppb)] produced significantly fewer byssus threads than control and low exposure animals.

5) No abnormalities were revealed by histopathological examination of selected adult mussel tissues. The lone exception was the appearance of inflammatory cells in spongy connective tissue surrounding the digestive gland.

6) Results of this study indicate that some physiological functions (e.g. byssus thread production) may be affected by bioaccumulated hydrocarbons and their effects on gamete viability remain unclear.

RECOMMENDATIONS FOR FUTURE RESEARCH

The experiments reported herein have clearly demonstrated that continuous exposure to a wide range (5 to 500 ppb) of petroleum hydrocarbon concentrations for periods ranging from weeks to months will result in significant mortality. In addition, these experiments have demonstrated adverse sublethal effects that may limit the reproductive success of exposed individuals and populations. Important sublethal effects can be evaluated in greater detail using a similar protocol, but with modifications in the duration of exposure, timing of exposure, and exposure concentration. We recommend experiments be conducted in which these variables are modified to simulate environmentally realistic conditions.

The objectives of future research should be to examine:

1) the sublethal effects of exposing embryos and larvae to low WSF concentrations for environmentally realistic time periods;

2) the sublethal effects of exposing embryos and larvae to low WSF concentrations during critical developmental periods;

3) the depuration mechanisms of embryos and larvae exposed to low WSF concentrations;

4) the effects of WSF exposure on gamete viability and the reproductive potential of previously exposed animals;

5) the sublethal effects of WSF on selected physiologic processes (e.g. byssus thread production quality and quantity) and the ecological consequences of these effects.

Selection of Test Oil, Exposure Concentrations, Exposure Durations and Exposure Timing

The original selection of Santa Barbara crude oil as the experimental toxicant in our experiments was based on the high probability of this oil entering the Pacific Coast marine environment, and the general applicability of experimental results obtained with its use. There is no reason to utilize another oil for future experiments since the original rationale still applies, and detailed characterizations of the parent oil and WSF test solution have already been conducted.

The target WSF concentrations selected for our present experiments were 5, 50, and 500 ppb. In general, the 500 ppb target concentration resulted in high levels of mortality in all experiments. Since the objective of our recommended studies is to investigate sublethal effects, we recommend experimental dosing at concentrations ranging between 5 and 50 ppb.

A key variable which we believe to be significant is the timing of toxicant exposure. Although the reproductive cycles of many fishes are relatively predictable in time and space, an event which releases hydrocarbons into the environment is not. Depending on the timing of a spill, hydrocarbon exposure may occur during any period of embryonic or larval development. Our experiments indicate that critical larval development periods are the: 1) embryonic stage; 2) yolk-sac stage (non-feeding); 3) yolk-sac absorption

and the initiation of first feeding and 4) the structural development of early feeding larvae. The experiments outlined below reflect consideration of these critical developmental periods in addition to the exposure duration.

In addition, proposed adult experiments reflect concern for future generations of animals in terms of reproductive success and physiological fitness.

Selection of Test Organisms and Life Stages

1) Experiments should be conducted with gametes of northern anchovy and California halibut to clearly define the effects of WSF exposure during fertilization on (a) fertilization success, (b) subsequent hatching success, and (c) subsequent larval survival.

2) Experiments should be conducted with California halibut and northern anchovy embryos to determine the effects of exposure to WSF petroleum hydrocarbons from soon after fertilization until hatching is completed [approximately 2 days) on subsequent hatching success, mortality, development and deputation.

3) Recently hatched yolk-sac larvae of California halibut and northern anchovy should be exposed to WSF petroleum hydrocarbons until completion of yolk-sac absorption and the initiation of feeding (i.e. 4 days) to determine the post-exposure effects on growth and development of early feeding larvae, and subsequent deputation.

4) Experiments should be conducted with the embryonic and yolk-sac larval stages of California halibut and northern anchovy to determine the effects of WSF exposure. Following exposure larvae should be transfered to clear seawater for evaluation of deputation, survivorship, feeding and development.

5) Post-yolk-sac larval exposure experiment with California halibut, northern anchovy, or other species. Early feeding larvae having completed yolk-sac absorption should be exposed to WSF petroleum hydrocarbons for a short period (e.g. 7 days) and then transfered to clean seawater. Subsequent evaluation for survivorship, growth and development will follow.

6) Exposure experiments should be conducted with non-commercial species of fish to determine if the general responses to WSF exposure observed in our studies are similar. Candidate species include Genyonemus lineatus, Seriphus politus, Citharichthys stigmaeus, C. sordidus, and one or more atherinid species.

7) Avoidance experiments should be conducted with adult test organisms to determine their behavioral responses to WSF exposure.

8) Short-term, pulse exposure (i.e. 7 to 14 days) experiments should be conducted with adult test organisms. Exposure should be followed by a period during which recovery and deputation is monitored. Separate exposure studies should be conducted on both males and females, and during both reproductive and non-reproductive periods.

9) Gamete viability study with California mussels. Adult mussels having been exposed for selected periods of time to WSF petroleum hydrocarbons will be transferee to clean seawater and induced to spawn. Gamete viability, fertilization success and development will be evaluated.

10) Byssus thread production and strength evaluation in California mussels. Byssus threads produced by mussels exposed to acute and chronic doses of WSF will be examined. The strength and holding characteristics will be evaluated and related to ecological needs in natural situations.

11) In addition to parent hydrocarbons, tissue burdens from exposed organisms should be examined in terms of petrogenic hydrocarbon metabolizes. Recent studies suggest that hydrocarbon metabolism frequently results in formation of extremely reactive epoxides which may be detrimental to cellular function through enzyme inactivation. Examination of parent hydrocarbon tissue burdens may thus be an extremely conservative measure of toxicant uptake phenomena.

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Abscess — A localized collection of purulent exudate (pus) within tissue.

Acidophilic GranularC all — (AGA, Acidophilic Granular Amoebocyte). A leukocyte found mainly in the interstitial tissues of Mytilus and increasing in numbers at sites of inflammation.

Adenoma — A benign tumor of glandular tissue.

Adhesions — Abnormal fibrous attachment between surfaces. Fibrous bands (adhesions) may form between loops of bowel, or between loops of bowel and the parietal peritoneal surfaces, for example. Fibrous adhesions may form between secondary gill filaments following inflammation.

Agranulocytosis — Decrease in the number of circulating granulocytes, usually resulting from toxicity to a drug or chemical poison causing depression of hematopoetic tissue.

Aliphatic Hydrocarbon — Hydrocarbon compound which consists of chains of carbon atoms that do not. involve cyclic structures. The three major categories of aliphatic hydrocarbons are alkanes, alkenes, and alkynes.

Alkane — Aliphatic hydrocarbon in which each carbon atom is bonded to four other atoms.

Alkene — Hydrocarbons which possess one or more carbon-carbon double bonds.

Alkyl-substituted — General term used to indicate a compound in which one or more hydrocarbon groups has been attached to the parent compound.

Anemia — Deficiency in circulating red cells, or of hemoglobin, or both.

Aneurysm — Local dilatation of a blood vessel, usually an artery, but may also be a vein or capillary.

Angiitis — Inflammation of a blood or lymph vessel.

Anuria . . . . . — Absence of secretion of urine by the kidneys; usually due to acute chemical injury.

Aromatic Hydrocarbons	Cyclic hydrocarbons, usually composed of five or six-membered rings, which contain alternate single and double bonds.
Ascites	Free fluid in the peritoneal cavity.
Basophil	(polymorphonuclear basophil) A blood leukocyte having cytoplasmic granules which have an affinity for basic dyes.
Biogenic Hydrocarbon	Hydrocarbon compound derived from a biological source (plant or animal).
Byssus	Organic threads produced by the California mussel, which in nature attach the animal to underlying substrate.
Cachexia	Malnutrition and general ill health.
'Cancer	Any malignant tumor.
Capsule	The outer membranous covering of various normal organs, and some neoplasms.
Collagen	The main constituent of white fibrous tissue, and of scar.
Cyclic(or Alicyclic) Hydrocarbons	Hydrocarbon compounds composed of carbon atoms arranged in a ring or rings.
cyst	A sac with a membranous lining containing fluid.
Edema	Edema is the accumulation of watery fluid in tissue spaces. Edema may occur in any organ. Edema usually results from increased capillary permeability due to endothelial and/or epithelial damage caused by anoxia or injurious agents of chemical, physical or biological nature. In gills, edema is an active change most easily observed at the base of secondary lamellae. In severe cases of edema the respiratory epitheliums of the primary and secondary lamellae delaminates.
Engraulis mordax	Tne northern anchovy; a common pelagic, schooling fish (Family: Engraulidae) of the West Coast of North America that ranges from the Queen Charlotte Islands to Magdelena Bay, Baja California. It is of sport and commercial importance, particularly in the Southern California Bight, and served as a test species (embryo, larval, and adult stages) in this study.

Fatty Change

The appearance of fat within the parenchymal cells of a tissue; an example is fatty change of the liver. Also sometimes called fatty infiltration or fatty metamorphosis. The fat droplets are sufficiently large to displace nucleus and cytoplasm to the periphery of the cell.

Fat Vacuolation

Same as fatty change.

Fibrous

Fibrosis is healing by scar formation. Fibrous obliteration of the gill may occur, which destroys secondary lamellae and diminishes respiratory function. Scarring that occurs during healing of a necrotizing process may subdivide the liver parenchyma, causing nodular (cirrhotic) liver. Mounds heal by fibrosis. Fibrosis can occur in any tissue.

Gas Chromatography

An instrumental technique for analysis of organic analytes in which a gas-liquid partitioning process is used for temporal separation of individual molecules in a complex mixture of molecules. The output is a series of time-sequenced peaks in which the area under the curve is directly proportional to concentration.

Glycogen Depletion

Glycogen depletion of hepatocytes in the liver results in the loss of cytoplasmic vacuolation characteristic of stored glycogen. Glycogen depletion in stained tissue preparations is characterized by an increased staining intensity, and a loss of hepatocytic cytoplasmic foaminess. Glycogen depletion reflects a minimal degree of stress.

Heterocyclic Hydrocarbons

Cyclic hydrocarbons, usually composed of five or six members, in which one or more of the atoms in the ring is an element other than carbon.

Homolog

A succession of hydrocarbons which differ by only one entity or unit.

Hyaline

Glassy, eosinphilic material.

Hyaline Acidophil Bodies

Hyaline acidophil bodies are individually necrotic liver cells.

Hydrocarbon

Organic molecule containing the elements hydrogen and carbon exclusively.

Hyperemia (congestion) — This is increased blood in the vascular bed. Hyperemia correlates with gross redness of the tissue seen in acute inflammation.

Hyperplasia — Hyperplasia is an increase in the number of cells in any tissue. Hyperplasia of respiratory epithelial and mucous cells may occur as a reaction to low level chemical or other kinds of relatively prolonged irritation. In severe instances, epithelial bridges and cysts form between adjacent secondary lamellae.

Hypertrophy — Hypertrophy is enlargement or thickening of a cell or parts of an organ. Hypertrophy of primary and secondary gill lamellae may occur due to variable combinations of epithelial hyperplasia, edema, thrombosis, and fibrosis.

Hypoplasia — Faulty and retarded development of any tissue or organ.

Infarct — Necrosis of a tissue due to occlusion of its blood supply.

Inflammation — The basic reaction of living tissue to mechanical injury, infection, chemical irritation or ionizing radiation; characterized by redness (hyperemia), swelling, and loss of function. There are several kinds, distinguished by the type of inflammatory infiltrate.

Acute inflammation has cellular infiltrate dominated by neutrophils.

Chronic inflammation has a cellular infiltrate dominated by lymphocytes and plasma cells.

Granulomatous inflammation has an infiltrate dominated by macrophages.

Sub-acute inflammation is intermediate between acute and chronic inflammation.

Ischemia — Hypoxia of a tissue due to deficient blood supply.

Isomer — One of two or more molecules having the same kind and number of atoms, but differing with respect to the arrangement or configuration of the atoms.

Leukemia — Neoplasia of white blood cells.

Leukocytosis	increased number of circulating of white blood cells.
Mass Spectometry	An instrumental technique generally used in conjunction with gas chromatography for identification and quantitation or organic analytes. Mass spectrometry is a detection device which produces information regarding the molecular structure of the analyte of interest.
Melanin-Macrophage Centers (MMC)	MMC in liver are groups of histiocytes (Kupffer cells) which contain melanin. They vary in size and number and may be unusually large and numerous in livers damaged by toxic chemicals.
Microaneurisms (telangiectasis)	Microaneurisms are dilated segments of blood vessels; for example, microaneurisms of the secondary gill lamellae may result from rupture of the pillar cells which maintain uniform spacing between the dorsal and ventral epithelial layers of the secondary lamellae. Microaneurisms grossly appear as small red spots ("blood blisters") on the secondary lamellae. Microaneurisms may result from either chemical or physical damage.
Mytilus californianus	The California mussel, whose various life history stages were experimentally exposed to oil in this study. in nature, this organism occupies the exposed rocky Pacific coastline and ranges from Alaska to Baja California.
n-Alkane (normal-alkane)	Aliphatic hydrocarbon in which the carbon atoms are connected in a single continuous sequence.
Necrosis	Necrosis is cell death and may occur in any tissue. Necrosis in the gill usually effects secondary lamellar epitheliums which sloughs forming ulcers. An exudate of mucous, necrotic epitheliums, leukocytes, detritus and bacteria may form on the ulcerated zone causing impaired respiratory function. Necrosis in liver may be diffuse (massive) or focal.
Neoplasm	New growth, or tumor; neoplasms are phenotypically new phenomenon; the cells of neoplasms grow relatively independently of the tissue regulatory systems of the host.

Neutrophil	(polymorphonuclear neutrophil) A blood leukocyte having neutrophilic cytoplasmic granules, and involved as a major participant in acute inflammation.
Occlusion	Closure of an opening, or duct, or blood vessel.
Oncogenic	Capable of inducing tumor formation.
Oliguria	Deficiency of urine formation.
Paralichthys californica	The California halibut; a bottom-dwelling flatfish (Family: Bothidae) occurring from British Columbia to Magdelena Bay, Baja California. The species supports a valuable sport and commercial fishery throughout California, and served as a test species (embryo, larval, and adult stages) in this study.
Petrogenic Hydrocarbon	. Hydrocarbon compound of petroleum origin.
Polar Hydrocarbon	Hydrocarbon molecules which possess areas of relative positive and negative charge. Such molecules ionize to some degree in solution; and are thus relatively soluble in water (heterocyclic hydrocarbons are an example).
Prenoplasia	This term is applied to any pathological change believed or proven to be at increased risk of neoplastic (tumor) development. For example, nodular (cirrhotic) livers in fish give rise to hepatocarcinoma (liver cancer) more frequently than does normal liver.
Regenerative Nodules	Spherical formations of liver cells that are associated with fibrous septation often developing from necrosis. The resulting nodular liver is said to be cirrhotic.
Standard Length	Length of fish as measured from tip of snout to end of hypural plate at base of caudal fin.
Total Length	Length of fish as measured from tip of snout to tip of longest lobe of caudal (i.e. tail) fin.
Tumor	Same as neoplasm.
Benign	. . . A tumor that grows relatively slowly and is encapsulated and well differentiated.

Malignant — Tumor which grows relatively rapidly, is not encapsulated, and which invades surrounding tissues, or may metastasize (colonize) distant organs. Malignant tumor is synonymous with cancer.

Veliger — The larval form characteristic of bivalve mollusks such as the California mussel examined in this study. Veligers possess a symmetrical shell consisting of two halves. The body structures are enclosed by the shell.